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The role of polyglutamine aggregate cytotoxicity in Huntington's Disease

Wen Yang
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Wen Yang entitled "The role of polyglutamine aggregate cytotoxicity in Huntington's Disease." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Ronald B. Wetzel, Major Professor

We have read this dissertation and recommend its acceptance:

Karla J. Matteson, Nicholas T. Potter, Xuemin Xu

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Acceptance for the Council:

Anne Mayhew
Vice Provost and Dean of
Graduate Studies

(Original Signatures are on file in the Graduate Student Services Office.)

THE ROLE OF POLYGLUTAMINE AGGREGATE CYTOTOXICITY IN HUNTINGTON'S DISEASE

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Wen Yang

May 2003

DEDICATION

This dissertation is dedicated to:

my parents

Dexin Yang

杨德新

and

Huarong Zhang

张华荣

my husband

Yupeng Zhang

张育鹏

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ABSTRACT

Polyglutamine diseases refer to a group of neurodegenerative diseases, including Huntington's disease (HD), that share the same mutational basis: expansion of a CAG repeat coding sequence that encodes for a polyglutamine repeat in the respective proteins. Neuronal intranuclear inclusions (NII) formed by the aggregation of the expanded polyglutamine proteins is a hallmark of polyglutamine diseases. However, it is still under debate whether the polyglutamine aggregates are toxic to the cells, and if so, which aggregated forms are the toxic species. While important data has been provided by the existing transfected cell and transgenic animal models, technical aspects have limited our ability to rigorously interpret this data in terms of molecular and cellular mechanism. A number of comprehensive studies on *in vitro* polyglutamine aggregation kinetics, and features of the aggregates produced *in vitro,* provided the foundation to directly address the question of the cytotoxicity of polyglutamine aggregates. In the present study, we designed a new cell model by directly introducing *in vitro* synthesized polyglutamine aggregates into mammalian cells, both PC-12 and Cos-7, to study the cytotoxicity of different forms of aggregates. We found that cell viability was largely unaffected when polyglutamine aggregates are localized to the cytoplasm; aggregates of polyglutamine peptides containing a nuclear localization signal, however, are localized to nuclei and lead to dramatic cell death. This cytotoxicity is polyglutamine-sequence-specific. We also found that a short polyglutamine peptide (Q_{20}), once aggregated and delivered to the cell nucleus, seems to be equally cytotoxic. Further studies suggest that the mechanism of cell death in this novel model system seems to be apoptotic. Our results support a direct

role for polyglutamine aggregates in cytotoxicity and are consistent with a recruitment-sequestration disease mechanism. This is further supported by studies, also described here, in which a polyglutamine aggregation elongation inhibitor protects against cell death induced by nuclear polyglutamine aggregates.

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LITERATURE REVIEW

Polyglutamine diseases

Introduction

Polyglutamine diseases are a group of neurodegenerative disorders caused by the expansion of CAG repeats encoding for polyglutamine tracts in different affected proteins (Bates 2002). Since the discovery in 1991 of the expanded CAG repeat mutation in the androgen receptor gene (La Spada, Wilson et al. 1991), which is responsible for Spinal and Bulbar Muscular Atrophy (SBMA), a total of nine polyglutamine diseases have been discovered (Zoghbi and Orr 2000; Bates 2002). These include Huntington's Disease (HD) (Myers, MacDonald et al. 1993), Spinal and Bulbar Muscular Atrophy (SBMA) (La Spada, Wilson et al. 1991), Dentatorubral – Pallidoluysian Atrophy (DRPLA) (Koide, Ikeuchi et al. 1994; Nagafuchi, Yanagisawa et al. 1994) and several forms of Spinocerebellar Ataxia (SCA 1, 2, 3, 6, 7, 17) (Orr, Chung et al. 1993; Kawaguchi, Okamoto et al. 1994; Sanpei, Takano et al. 1996; David, Abbas et al. 1997; Zhuchenko, Bailey et al. 1997; Koide, Kobayashi et al. 1999). The discoveries of 9 polyglutamine diseases and the citations of the literatures are summarized in Table 1.

In the literature, polyglutamine disease is often called expanded CAG repeat diseases or disorders (Burright, Orr et al. 1997), (a) because most of polyglutamine

Table 1. History of the discovery and research on the 9 polyglutamine diseases

Diseases (abbreviation)	Year of gene discovered	Reference on the first discovery of the gene mutation	Number of the research papers since the discovery (from PubMed by Feb. 3, 2003)
HD	1993	Huntington's Disease Collaborative Research Group1993	1773
SBMA	1991	La Spada et al. 1991	206
DRPLA	1994	Koide et al and Nagafuchi et al. 1994	93
SCA1	1993	Orr et al, 1993	81
SCA2	1996	Sanpei et al. 1996	50
SCA3	1994	Kawaguchi et al. 1994	33
SCA6	1997	Zhuchenko et al. 1997	34
SCA7	1997	David et al. 1997	26
SCA17	1999	Koide et al. 1999	3

sequences in the mutant proteins are coded by a simple, unbroken CAG repeat DNA sequence (Bates 2002) (except for in SCA2 and SCA17, in which repeats were interrupted with 1-2 CAA, which also codes for glutamine (Costanzi-Porrini, Tessarolo et al. 2000; Fujigasaki, Martin et al. 2001)); and (b) because a genetic expansion of this sequence from a normal, benign length to a pathogenic length is associated with disease (Bates 2002).

From genetic aspects, polyglutamine diseases belong to a large group of hereditary diseases called trinucleotide repeat diseases. To date, there are more than 15 neurological disorders discovered in this group, including the polyglutamine diseases (Cummings and Zoghbi 2000). Expansion of a trinucleotide repeat can occur in any part of a gene, in-frame or not. In the nine polyglutamine diseases, however, the expanded trinucleotide repeat always lies in-frame in the coding region of a gene, which results in normal transcription and translation of the genes to yield a protein with an expanded polyglutamine sequence. At the cellular and molecular level, most of the evidence so far suggests that these diseases are caused by a toxic gain of function due to the expansion of the polyglutamine sequence in the affected proteins (Wilmot 1998; Bates 2002). The strongest evidence for this conclusion is that expression of a CAG repeat expansion within the context of the unrelated protein *hprt* causes a similar neurodegenerative disease (Ordway, Tallaksen-Greene et al. 1997). This suggests that expansion of polyglutamine itself is responsible for the neurodegeneration, and further suggests that the protein context of different disease proteins probably play roles in defining the specificities of different diseases. Recent studies in HD suggest that some partial loss of

function of the huntingtin protein may also contribute to disease onset (Cattaneo, Rigamonti et al. 2001). Nonetheless, polyglutamine diseases often present as a dominant inherited pattern, except for SBMA (this will be explained in SBMA section) (Zoghbi and Orr 2000). In the other trinucleotide repeat diseases, mutations occur in 3 ', 5' untranslated regions (UTR) or introns; these mutations interfere with normal gene transcription or translation and result in partial or complete depletion of the gene products. This usually leads to a loss of function mutation and presents as a recessive inheritance pattern (Reddy and Housman 1997). In Figure 1, the different trinucleotide repeat diseases are illustrated according to where in the gene the mutation occurs.

The discovery of trinucleotide repeats expansion in 1991 (La Spada, Wilson et al. 1991) revealed a new mutational mechanism, called dynamic mutation (or genetic instability), which is characterized by distinct inheritance features, such as increasing penetrance or anticipation (increasing disease severity and decreasing age of onset in successive generations due to increasing lengths of trinucleotide repeats in an affected family) (Wilmot 1998; Usdin and Grabczyk 2000). Mechanisms of repeat expansion are poorly understood. Based on *in vitro* bacteria and yeast studies, mechanisms of repeat expansion can be explained by invoking aspects of DNA structure, replication and repair (Wilmot 1998). First, repetitive DNA can adopt other more stable structures than double helix, such as hairpin, triplex and tetraplex (Kovtun and McMurray 2001) and these structures stabilize the repeats and interfere with normal DNA replication and repair. Second, expansions can occur by strand slippage during DNA replication (Schweitzer and Livingston 1999) and/or by misalignment during DNA excision repair

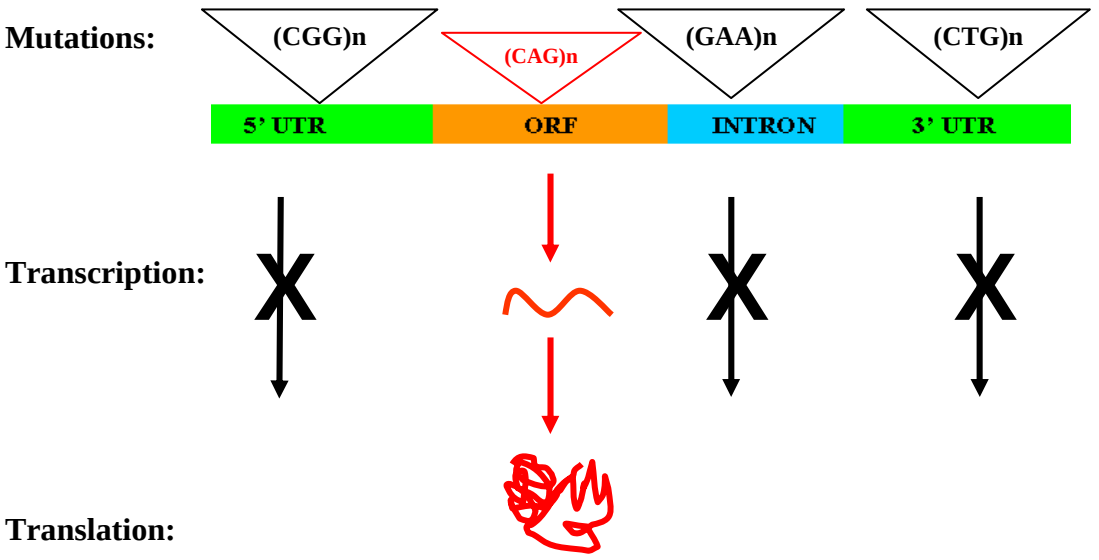


Figure 1. Features of trinucleotide repeat diseases, including polyglutamine diseases

(Schweitzer and Livingston 1997) and recombination of DNA strands (Jakupciak and Wells 1999). Detailed hypotheses of the expansion mechanism have been reviewed (Usdin and Grabczyk 2000; Kovtun and McMurray 2001).

From clinical and pathological points of view, polyglutamine disease can be linked with a large family of neurodegenerative diseases featuring the presence of aggregates or inclusion bodies in brain tissue (Taylor, Hardy et al. 2002); these include Alzheimer's disease, Parkinson's disease, prion diseases, Amyotrophic Lateral Sclerosis (ALS) and the tauopathies. The aggregated proteins and the location of these aggregates in the brain tissue are different for different diseases (Merlini, Bellotti et al. 2001). At the same time, protein misfolding and aggregation can be considered to be fundamental problems underlying all these diseases (Taylor, Hardy et al. 2002). Table 2 summarizes the aggregation related neurodegenerative diseases.

None of the nine polyglutamine diseases are curable at the current time. At present, the only available medical treatment is the care and supportive therapy that are used in the clinic (Kieburtz 2002). Within families, the severity of disease tends to increase in successive generations (as described by anticipation, a hallmark of these diseases). Offspring tend to inherit even longer repeat expansions (especially through paternal transmission) due to the genetic instability imparted by the repeat length (Wilmot 1998). Since there are many other known CAG repeats coding for polyglutamine containing proteins in the genome that have not been linked to a disease, and given that a repeat expansion mutation might occur in virtually any of those proteins, one can imagine that there will be more polyglutamine diseases discovered in the future

Table 2. Protein aggregation and neurodegenerative diseases [a]

Disease	Disease genes	Responsible protein	Protein aggregates
Polyglutamine Diseases	CAG repeat expansion lies in different genes	Polyglutamine containing proteins	Neuronal intranuclear inclusions (NII)
Alzheimer's Disease	APP, Presenilin1, Presenilin2	Aβ/tau	Extracellular plagues (by Aβ) /intracellular tangles (by tau)
Parkinson's Disease	α-Synuclein, Parkin	α-Synuclein	Lewy bodies
ALS	SOD1 [b]	SOD1	Bunina bodies
Prion Diseases	PRNP	PrP^{Sc}	Prion plaques
Tauopathy	tau	tau	Cytoplasmic tangels

[a] The table was modified from (Taylor, Hardy et al. 2002).

[b] The relationship of familial ALS involving SOD1 mutations, to other form of ALS, is not clear.

(Cummings and Zoghbi 2000). The situation is devastating. Hope for curing the disease in the future depends on the emergence of a better understanding of the common mechanisms of all polyglutamine diseases that will ultimately lead to the discovery for a cure.

In this chapter, I will first introduce each of the nine known polyglutamine diseases from historical, epidemiological, clinical, neuropathological, genetic and molecular aspects. Then, the diseases will be compared with respect to their similarities and differences, in order to bring out the possibility of a unified mechanism behind all of the polyglutamine diseases. The third part will be a review of the discoveries drawn from the studies of the existing experimental model systems in polyglutamine disease, with brief discussions on the advantages and disadvantages of these models. Lastly, I will summarize the various proposed molecular and cellular mechanisms of the polyglutamine diseases, including the existing questions and controversies rising out of different studies. This will also include a discussion of various cell death mechanisms, including apoptosis, necrosis and other cell death mechanisms. This information provides critical background for understanding the goals, experimental design and conclusions of the experiments described here.

Huntington's Disease (HD)

History and epidemiology of HD

HD was first described in 1872 by Dr. George Huntington as "hereditary chorea" in his paper called *On chorea* (Huntington 1872). Together with the documentation from his father and grandfather, he observed a disease inherited in the successive generations of three families in Long Island, NY. HD is the most common and extensively studied disease among nine polyglutamine diseases. The prevalence of HD in Caucasians is about 5-10 per 100,000 individuals (Harper 1992). HD is less common among Asian population (1/10 of the Caucasians) and very rare in African population (Harper 2002).

Clinical manifestations of HD

The characteristic clinical manifestation of HD is chorea, which is a Greek word meaning *dance*. A more precise clinical definition for chorea is "a state of excessive, spontaneous movements, irregularly timed, randomly distributed and abrupt" (Barbeau, Duvoisin et al. 1981). In the early state of HD, chorea can be presented as fasciculations (muscular twitching of contiguous groups of muscle fibers) of facial muscles or random twitching of the fingers and toes. As the disease progresses, it will affect larger movements of the arms, legs and whole body, and present as "dance-like gait" and ballismus (a type of involuntary movement affecting the proximal limb musculature, manifested as flinging movements of the extremity) in some severe cases. Other than chorea, there are also other motor dysfunctions present, such as dystonia (disordered

muscle tone), hyperactive reflexes and speech abnormalities (Kremer 2002). Along with these movement disorders, HD patients often have different degrees of cognitive and psychiatric symptoms, such as memory loss and personality changes (Craufurd and Snowden 2002). HD is mainly a midlife-onset (40 ±10 years of age) disease, but can start at any age (2-80 years of age). HD is ultimately fatal with an average of 15-17 years after the onset of symptoms (Kremer 2002).

Neuropathology of HD

The neuropathological hallmark of HD at the macroscopic level is the striking atrophy of the neostriatum, in which the loss of caudate nucleus is the most dramatic: up to 95% of the neurons of the caudate nucleus are lost in the late stage of HD, accompanied by an overall brain atrophy (Heinsen, Strik et al. 1994; Gutekunst, Norflus et al. 2002). Figure 2 is a postmortem brain specimen showing the brain atrophy at the different stages of HD compared to a normal aged control brain (Myers 1998). Figure 3 is a schematic illustration of the brain pathology of HD and some other polyglutamine diseases derived from a review of Bates *et al*. (Bates 2002). Brain weight is often reduced by about 10-20% in HD compared to a normal control brain measured at autopsy (Gutekunst, Norflus et al. 2002).

At the cellular level, it has been found that the medium size striatal neurons in postmortem HD brain are selectively depleted compared to large staital neurons (Heinsen, Strik et al. 1994). These medium spiny neurons are inhibitory projection neurons of striatum and the major component (95%) of the caudate nucleus (Gutekunst, Norflus et al. 2002). This selective loss of medium striatal neurons may underlie the chorea movement

Figure 2. Comparasion of HD brain atrophy with normal aged control brain.

Figures shown here are the hemicoronal sections of postmortem brain from (I) normal aged control, (II) early state of HD (grade 2), (III) mid state of HD (grade 3) and (IV) late state of HD (grade4). One can appreciate from these figures how dramatic the brain atrophy in HD is. Figure is taken from (Myers 1998).

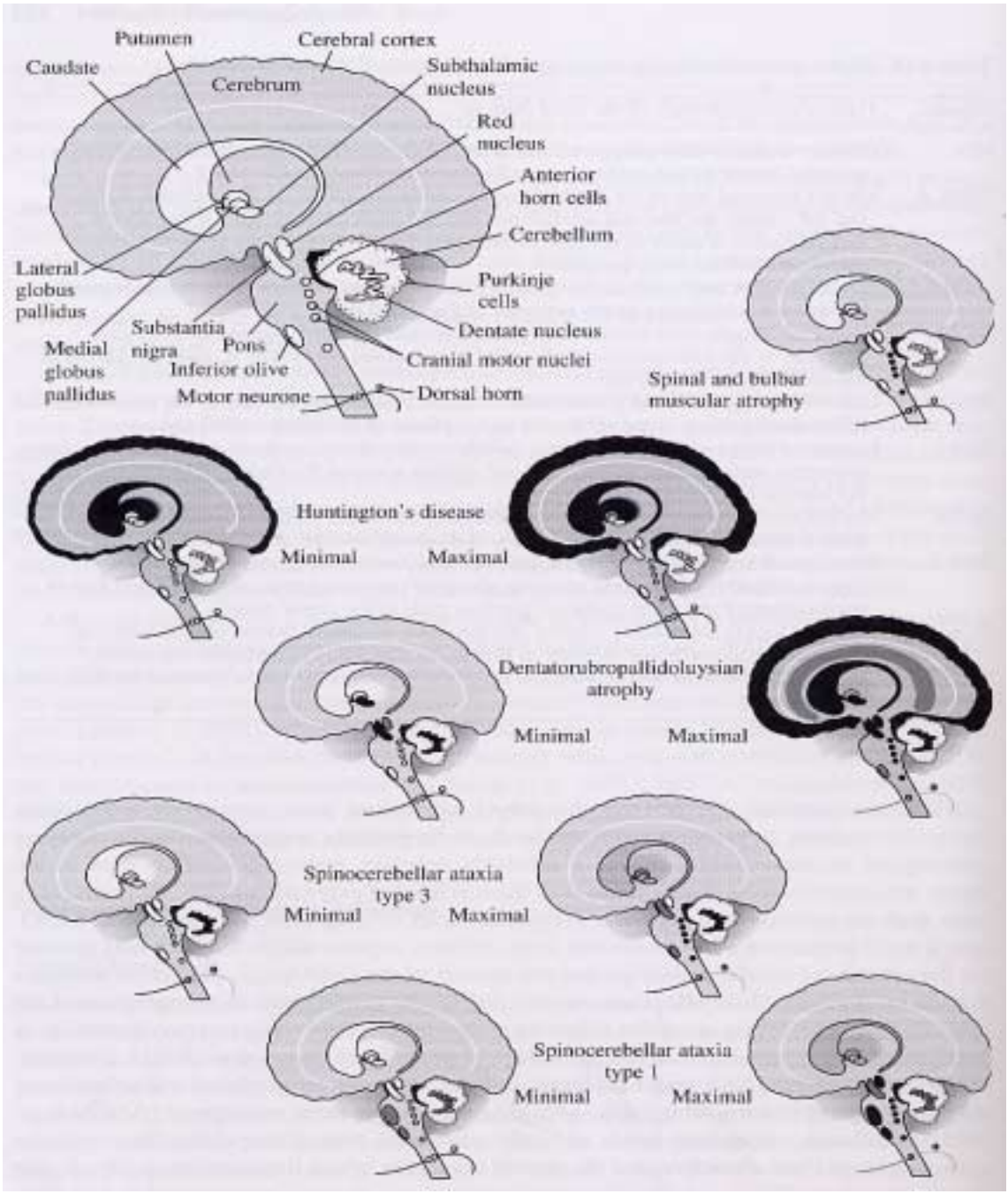


Figure 3. Schematic depiction of the brain pathology of 5 polyglutamine diseases.

The affected areas are indicated by shading (the darker, the more severely affected). From (Bates 2002).

in patients (Heinsen, Strik et al. 1994). Organs other than brain are largely unaffected, even though the HD gene is expressed universally (Sharp, Loev et al. 1995).

At the subcellular level, in both striatum and cortex, neuronal intranuclear inclusion (NII) bodies are characteristic to HD (DiFiglia, Sapp et al. 1997). NIIs are protein aggregates made up mainly of N-terminal fragments of mutant huntingtin. Ubiquitin, α-synuclein, cAMP-response element binding protein [(CREB)-binding protein or CBP] have also been shown to be recruited into the aggregates (DiFiglia, Sapp et al. 1997) (Nucifora, Sasaki et al. 2001). NIIs are large, 3-5um in diameter, and mostly round in shape, with only 1 or 2 per nucleus (DiFiglia, Sapp et al. 1997). Figure 4 is an electron micrograph showing the NII in the neuronal nucleus of a postmortem HD brain (DiFiglia, Sapp et al. 1997). Extra-nuclear aggregates, such as perinuclear (on the nuclear exterior surface) or neuropil (axonal, dendritic and synaptic) aggregate, can also be found in the HD brain (Gutekunst, Norflus et al. 2002). They are usually smaller in size and larger in number compare to NII. Studies have shown that some of these aggregates can be stained with Congo red and exhibit birefringence under polarized light microscope, and also possess filamentous ultra-structures under electron microscopy (McGowan, van Roon-Mom et al. 2000), suggesting it is an amyloid-like structure. Increased DNA fragmentation detected in HD striatal neurons suggests that apoptosis is involved in the cell death mechanisms (Butterworth, Williams et al. 1998), while gliosis has also been observed near affected neurons (Gutekunst, Norflus et al. 2002), suggesting that necrosis may also be involved.

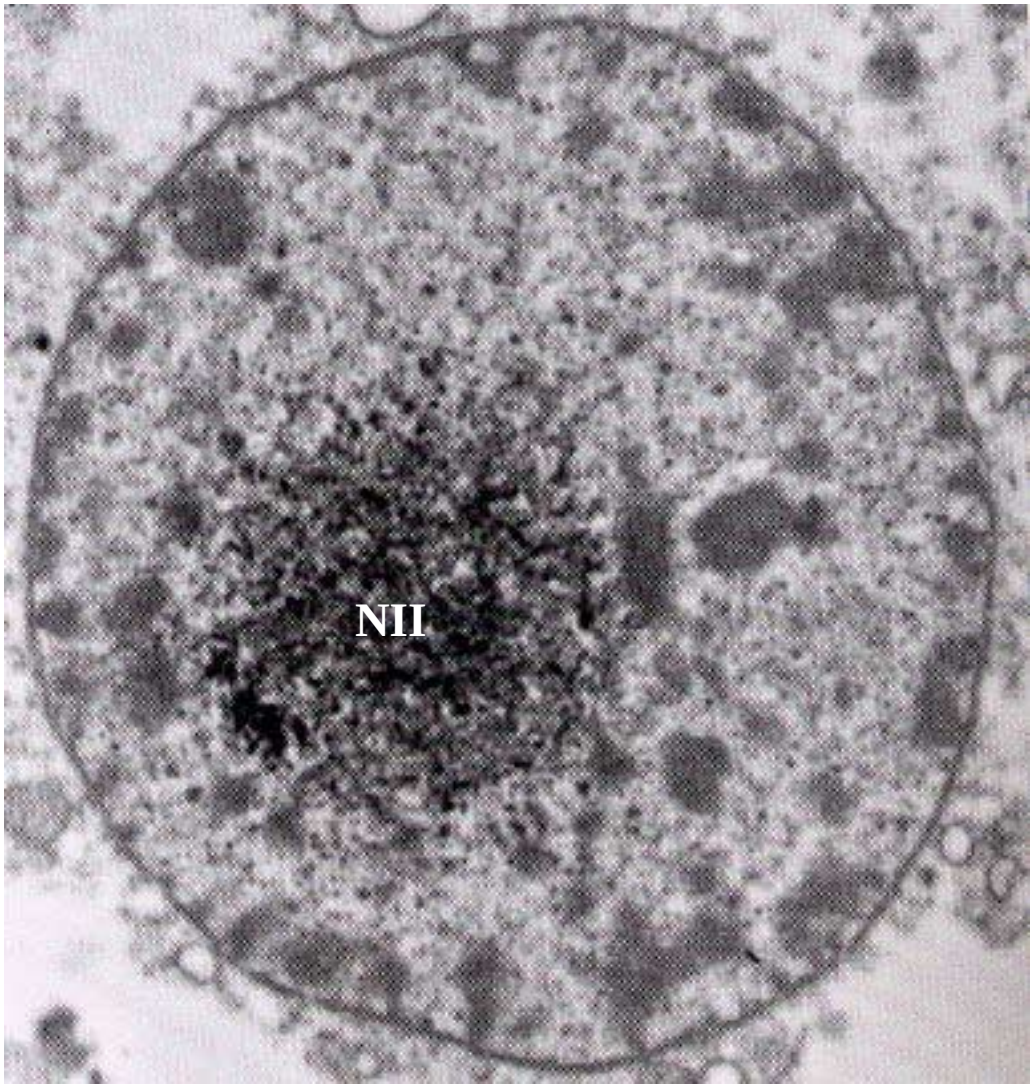


Figure 4. Electron micrograph of the NII in the nucleus of postmortem HD brain.

(Taken from (DiFiglia, Sapp et al. 1997))

Genetics of HD

The inheritance pattern for HD is clearly autosomal dominant (Gusella and MacDonald 1995). In 1983, the HD gene was mapped to chromosome 4p16.3 by genetic linkage analysis. After 10 years of molecular genetic analysis, the HD gene and mutation were finally revealed in 1993 by a large group effort (Huntington's Disease Collaborative Research Group) (Myers, MacDonald et al. 1993). The HD gene is about 185 kb and contains 67 exons. An expanded CAG repeat was found in exon 1 of HD gene in HD patients, coding for a polyglutamine tract near the N-terminus of the predicted gene product, called huntingtin. Unaffected individules have a repeat length ranging from 6 to 39, while HD patients have repeat lengths ranging from 36 to over 200 (Bates 2002).

Protein product and normal function

Huntingtin is a large (around 350 kD) cytoplasmic protein of unclear function. It is widely expressed throughout the brain and in other tissue. In brain, huntingtin is associated with microtubules in dendrites and synaptic vesicles, suggesting it may have some function in synaptic transportation and transmission (Sharp, Loev et al. 1995). There are two proteins known to interact with normal huntingtin. One is huntingtin-associated protein 1 (HAP1) (Li, Li et al. 1995; Peters and Ross 2001) and the other is Huntingtin-interacting protein 1 (HIP1) (Kalchman, Koide et al. 1997; Wanker, Rovira et al. 1997). Their functions are not very clear. Some studies suggest that huntingtin may be involved in intracellular trafficking, vesicle transmission and endocytosis, further supporting the hypothesis that huntingtin may be associated with synaptic functions.

Recent studies also suggest that wide-type huntingtin may have a role in anti-apoptosis by depletion of a possible proapoptotic protein, HIP1 (Hackam, Yassa et al. 2000; Rigamonti, Bauer et al. 2000).

Spinal and Bulbar Muscular Atrophy (SBMA)

History and epidemiology of SBMA

W.R. Kennedy was the first to described SBMA, in 1968 (Kennedy, Alter et al. 1968), and the disease is often called Kennedy's Disease. In 1991, an expansion of a CAG repeat in the first exon of the androgen receptor gene was identified as the disease mutation (La Spada, Wilson et al. 1991). Thus, SBMA was actually the first recognized polyglutamine disease. The prevalence of SBMA is very low; an incidence estimate hasn't been reported. Gathering the information from OMIM (Online Mendelian Inheritance in Man at www.ncbi.nlm.nih.gov), there are less than 200 reported families diagnosed with SBMA world wide, from Finland, Sweden, Norway, Denmark, Germany, Belgium, Italy, Japan, Australia and Canada.

Clinical and neuropathological features

SBMA presents an X-link recessive inheritance pattern, with males predominantly affected. Female carriers exhibit mild symptoms under careful examination (Sobue, Hashizume et al. 1989). Despite the recessive inheritance pattern, SBMA is a dominant mutation at the molecular and cellular level. It is probably due to (a) random inactivation

of the X-chromosome that some heterozygous females don't have severe symptoms; (b) even if mutant AR is expressed in the female, the level is very low and may have little or no effect (Zoghbi and Orr 2000). The onset of the symptoms usually starts at around 30-50 years of age (there are also extreme cases reported as early as 12 years or late as 84 years), with muscle cramps at an early stage developing into muscle weakness and atrophy in proximal muscles like arms and shoulders. Fasciculation can occur in tongue and jaw muscles, with later development into dysarthria (difficulty in articulating words, caused by impairment of the muscles used in speech) (MacLean, Warne et al. 1996). In the male SBMA patients, testicular atrophy and subsequently infertility (described as androgen insensitivity) can also be observed, suggesting there is some partial loss of function (Zoghbi and Orr 2000). As the disease name suggests, the neuropathology is mainly the loss of motor neurons in the spinal cord and brain stem bulbar region (see Figure 3). The degeneration of anterior horn neurons in the spinal cord is severe, while the posterior horn cells are well preserved. Loss of primary sensory neurons in dorsal root ganglia has also been reported. The muscle atrophy is mainly the result of chronic denervation due to the loss of anterior horn spinal neurons (Zajac 1998). NIIs made of mainly ubiquitinated full length or cleaved AR are presented in motor neurons of SBMA patients (Li, Miwa et al. 1998).

Androgen receptor gene and protein product

The androgen receptor (AR) gene, located on chromosome Xq11-12, is over 100kb in size, and contains 8 exons coding for a protein of 919 amino acids (Lubahn, Joseph et al. 1988). The CAG repeat is located in the first exon of the AR gene. While the

normal repeat length is 9-33, in SBMA it expands to 38-65. AR is a steroid hormone receptor and a transcription factor that responds to androgen binding (Lumbroso, Lobaccaro et al. 1997). The structure of the AR protein is composed of a hormone binding site, a DNA binding domain, a nuclear localization signal (NLS) and a transcriptional activation region (Zajac 1998). The polyglutamine tract is in the N-terminus of AR. The complete depletion of polyglutamine does not affect the normal transcriptional function of AR; expanded polyglutamine slightly decreases AR activity and may account for the androgen insensitivity in SBMA. Complete loss of AR results in testicular feminization but not SBMA (Zoghbi and Orr 2000).

Dentatorubral – Pallidoluysian Atrophy (DRPLA)

History and epidemiology of DRPLA

Sporadic cases of DRPLA were originally described by Smith et al. (Smith 1975), while the hereditary form of DRPLA was first described by Naito *et al.* in *Neurology* in 1982 (Naito and Oyanagi 1982). DRPLA is mainly seen in Japan, with a prevalence of around 6 in 1,000,000 (Kondo 1998) A few cases of DRPLA have been reported outside of Japan, in Europe (Nielsen, Sorensen et al. 1996) and in the United States (Potter, Meyer et al. 1995; Potter 1996). In addition, Haw River Syndrome, described in an African-American family residing near Haw River in North Carolina, was later found to be caused by the same mutation of DRPLA (Vance 1998) and is now considered to be another form of DRPLA. The mutation of the gene responsible for DRPLA was

discovered by two independent groups in 1994 (Koide, Ikeuchi et al. 1994; Nagafuchi, Yanagisawa et al. 1994).

Clinical and neuropathological feature

The onset of DRPLA can range from 1 to 74 years of age, with a mean of about 27-33. Clinical features of DRPLA vary in each patient, with key features including ataxia (failure of muscular coordination, irregularity of muscular action), chorea and dementia, making the disease easily confused with HD prior to characterization of the mutation. In addition, some patients may have myoclonus (twitching or spasm of a muscle or a group of muscles), tremor, epilepsy (neurological disorders characterized by sudden recurring attacks of motor, sensory, or psychic malfunction with or without loss of consciousness or convulsive seizures) and psychiatric symptoms (Naito and Oyanagi 1982). The disease is usually fatal with a mean period between onset and death of 12 years (Kondo 1998). The major neuropathology of DRPLA is, as the disease name suggests, a combined atrophy in the dentatorubral and pallidolusian systems, including neuronal loss in the cerebral cortex, globus pallidus, striatum, cerebellar cortex, the dentate nucleus and the subthalamic and red nuclei, as shown in Figure 2. Gliosis and even calcification often accompany neuronal loss in some severe cases (Kondo 1998).

Mutation and gene products

The gene for DRPLA is on 12p13.31 and spans 20kbp with 10 exons. The CAG repeat is located in exon 5, 1462bp downstream from the start codon (Koide, Ikeuchi et al. 1994; Nagafuchi, Yanagisawa et al. 1994). The normal range for the repeat is about 3-35,

while the expanded repeats in DRPLA range between 49 and 88 (Bates 2002). The gene codes for a protein, called atrophin-1, containing 1185 amino acids (Yazawa, Nukina et al. 1995). The function of atrophin-1 is not clear. Atrophin-1 contains a proline-rich region and several arginine-glutamic acid (RE) repeats, which may have some function in protein interaction (Yanagisawa, Bundo et al. 2000). The drosophila homolog of atrophin-1 has been suggested to have transcriptional corepressor roles in multiple developmental processes of drosophila (Zhang, Xu et al. 2002). Atrophin-1 is widely expressed and mainly located in the cytoplasm of the neurons (Schilling, Wood et al. 1999). Nuclear inclusion bodies containing mutant atrophin-1 have been found in the affected neurons (Zoghbi and Orr 2000).

The Sprinocerebellar Ataxias

Spinocerebellar ataxias (SCAs), also called the autosomal dominant spinocerebellar ataxias (ADCAs), are a class of heterogeneous neurodegenerative disorders with different degrees and combinations of progressive degeneration of the cerebellum, brain stem and spinal cord, resulting in different degrees of progressive cerebellar ataxias (Stevanin, Durr et al. 2000). It was impossible to classify and distinguish each of the diseases in this family before the genetic markers of each disease were known. Based on clinical manifestations, ADCAs have been divided into three groups since 1983 (Klockgether and Evert 1998). With the discovery of the genes and mutations, each of ADCAs was given an individual name from SCA1 to SCA17. The overall prevalence of all the ADCAs together has been estimated to be about 10/100,000

(Stevanin, Durr et al. 2000). Certain SCAs may have very high prevalence in certain areas due to the founder effect; for example, SCA2 is 4/10,000 in Cuba, where the disease was discovered (Orozco, Estrada et al. 1989; Gispert, Nothers et al. 1993). In the 17 known SCAs, 6 have been discovered to feature an expansion of a CAG repeat in the respective genes as the mutational mechanism of disease. These are SCA1, 2, 3, 6, 7, and 17 (Stevanin, Durr et al. 2000). They will be introduced individually below.

Spinocerebellar Ataxia 1 (SCA1)

History of SCA1

In the early days, SCA1 was often confused with Friedreich's ataxia based on the clinical findings. In 1974, Yakura described a dominant inherited ataxia that later became known as SCA1 (Yakura, Wakisaka et al. 1974). By classic linkage analysis in a large family, Jackson *et al* later mapped the gene for the disease to chromosome 6p (Subramany SH 1998). In 1993, the gene mutation for SCA1, an in-frame expanded CAG repeat, was reported by Orr *et al*. (Orr, Chung et al. 1993)

Clinical manifestations and neuropathology

SCA1 is an autosomal dominant, progressive neurodegenerative disorder with the key clinical symptoms including ataxia, dysarthria, motor dysfunction, dysphagia (difficulty in swallowing) and amyotrophy (a type of neuropathy that causes muscle weakness and wasting). The age of onset of the disease ranges from 6 to 74 with a mean

age of around 35. The time between clinical presentation and death ranges from12 to 38 years. The mean death age has been estimated at 54 (Subramany SH 1998). The neuropathology is characterized by cerebellar atrophy with severe loss of Purkinje cells. Neurodegeneration has also been found in inferior olive neurons, dentate nucleus and brain stem (see Figure 3). At the cellular level, a single, large nuclear inclusion body is often observed in the Purkinje cells and brain stem neurons in SCA1 postmortem brain specimens (Cummings, Orr et al. 1999).

Gene product and protein function

SCA1 gene is located on chromosome 6p23, spans 450 kb of DNA and is composed of nine exons (Banfi, Servadio et al. 1994). The CAG repeat lies in the coding region of the gene and codes for a polyglutamine tract. In unaffected individules, the repeat length is about 6-44, with 1-3 CAT (coding for histidine) interruptions in the longer repeats (over 21). In SCA1, the repeat usually contains 39-83 pure CAG. The CAT codons in normal genes appear to play a role both in suppressing genetic repeat expansions and, in longer, normal range repeats, suppressing aggregation of the gene product (Matsuyama, Izumi et al. 1999). The gene product of SCA1 is a protein called ataxin-1, which is an 816-amino acid protein with a molecular weight of 87 kD (Banfi, Servadio et al. 1994). The polyglutamine tract is located at amino acid number 197. Ataxin1 is expressed both in CNS and peripheral tissues including heart, skeletal muscle, liver, pancreas and lung (Servadio, Koshy et al. 1995). The expression level in CNS is 2 to 4 times higher than that of other tissue, suggesting a neuronal function of the protein. In brain, the expression of SCA1 does not seem to differ qualitatively from region to

region. Ataxin1 has been found in both the cytoplasm and nucleus in the neurons, but only in the cytoplasm in the peripheral tissues (Servadio, Koshy et al. 1995). The exact function of ataxin1 is largely unknown. Studies in gene knock-out mice suggest it may have some function in learning and memory (Matilla, Roberson et al. 1998). *In vitro* studies show that ataxin1 has RNA-binding ability and may thus have a role in RNA metabolism at the cellular level (Yue, Serra et al. 2001).

Spinocerebellar Ataxia 2 (SCA2)

History of SCA2

The clinical description of SCA2 was first documented in a European family by Boller and Segarra in 1969 (Boller and Segarra 1969). SCA2 represent 13-15% of ADCA and the majority of SCA1 patients are from Caucasian ancestry.[Pulst, 1998 #2358] In 1993, the SCA2 gene was mapped to chromosome 12 (Gispert, Twells et al. 1993) by linkage analysis in a large Cuban kindred. Soon thereafter, the SCA2 gene was discovered and an expansion of its CAG repeat was identified as the disease causing mutation by positional cloning (Pulst, Nechiporuk et al. 1996), and by a new method called DIRECT (Direct Identification of Repeat Expansion and Cloning Techniques) (Sanpei, Takano et al. 1996).

Clinical manifestation and neuropathology

The clinical manifestations of SCA2 are similar to other SCAs, with ataxia, dysarthria and other spinocerebellar symptoms, in addition to which eye movement abnormalities have often been found in some patients.[Pulst, 1998 #2358] The age of onset ranges from 2 to 65 years.[Pulst, 1998 #2358] The major neuropathology findings are atrophy in the cerebellum and brain stem with severe loss of cerebellar Purkinje cells and granule cells. Neurodegeneration can also be found in the frontotemporal lobes and substantia nigra. In SCA2 brains, cytoplasmic aggregates and a diffuse pattern of nuclear staining (that might be microaggregates) have been found in the affected brain region (Koyano, Uchihara et al. 1999; Huynh, Figueroa et al. 2000).

Gene, mutation and protein

The SCA2 gene, located on chromosome 12q24.1, contains 25 exons and spans approximately 130 kb. The CAG repeat, interrupted by two CAA codons, is located in exon 1. The normal repeat size ranges from 13 to 33, while the SCA2 length ranges from 32 to over 200; the particularly long repeats cause severe infantile–onset SCA2 (Mao, Aylsworth et al. 2002). The SCA2 gene is widely expressed, including in the brain, heart, placenta, liver, skeletal muscle and pancreas (Imbert, Saudou et al. 1996). The gene product, called ataxin-2, is a 140kD protein with a polyglutamine stretch near the N-terminus. Normal ataxin-2 is located diffusely in the cytoplasm.[Pulst, 1998 #2358] The function of ataxin-2 is not clear, but it may be involved in developmental regulation, as studies in C-elegans identified an orthologue of ataxin-2 that is important for early

embryonic patterning (Kiehl, Shibata et al. 2001); and the mouse SCA2 gene homologue is expressed strongly in early embryogenesis (Nechiporuk, Huynh et al. 1998). Recent studies suggest that it might have some function in RNA splicing, since it is co-localized with a novel protein A2BP1 containing RNA-binding motifs (Shibata, Huynh et al. 2000).

Spinocerebellar Ataxia 3 (SCA3)

History of SCA3

SCA3 is also called Machado-Joseph Disease (MJD), named after the Machado and Joseph families, who were first reported with the clinical features of SCA3 in 1972 and 1976 respectively (Nakano, Dawson et al. 1972; Rosenberg, Nyhan et al. 1976). The prevalence of SCA3 is about 30 to 80 percent of the prevalence of all SCAs in different countries (Bates 2002). The SCA3 gene and mutation was discovered in 1994, based on linkage analysis in a Japanese SCA3 family (Kawaguchi, Okamoto et al. 1994) and a cytogenetic method (FISH) with a putative fragment probe (Takiyama, Nishizawa et al. 1993; Takiyama, Oyanagi et al. 1994).

Clinical manifestation and neuropathology

The major clinical manifestations of SCA3 include ataxia, dysarthria, Parkinsonism (a group of neurological disorders characterised by decreased muscular activity, tremor and muscular rigidity), dystonia and ophthalmoplegia (loss or impairment of the motor function of the ocular muscles) (Stevanin, Durr et al. 2000). The age of

onset of SCA3 varies from the teens to 60 years of age. The major neuropathological features are the degeneration of basal ganglia, globus pallidus, brain stem motor neurons, spinal cord and dentate nucleus (Stevanin, Durr et al. 2000), shown in Figure 2. The degeneration is mild in cerebellar Purkinje cells. In SCA3 patients, nuclear aggregates containing ataxin3, ubiquitin, and various components of the proteasome and cellular chaperones have been detected in the affected regions of brain (Paulson, Perez et al. 1997).

Gene, mutation and protein

The SCA3 gene is located on chromosome 14q32.1. The gene of SCA3 contains 48,240 bp and to contain 11 exons (Ichikawa, Goto et al. 2001). The CAG repeat lies in the coding sequence near the 3' end of the gene. Normal repeat lengths range from 3 to 40. In SCA3, the repeat length is 54-89 (Bates 2002). The gene product of SCA3 is a protein called ataxin-3, which is a 42kDa protein with unknown function. Ataxin-3 is widely expressed throughout the body and has no regional specificity in brain (Paulson, Das et al. 1997). In the neurons, ataxin-3 has been detected in both the cytoplasm and the nucleus and contains a putative nuclear transportation signal (NLS) near the polyglutamine stretch (Tait, Riccio et al. 1998). A recent yeast two-hybrid study revealed two human homologs of the yeast DNA repair protein RAD23 interacting with ataxin3, suggesting a nuclear function (Wang, Sawai et al. 2000).

Spinocerebellar Ataxia 6 (SCA6)

History of SCA6

The discovery of SCA6 was by an unusual route compared to that of the previous 6 polyglutamine diseases. It was already known that a polymorphic CAG repeat lay within the human alpha 1A voltage-dependent calcium channel subunit (CACNA1A) (Ophoff, Terwindt et al. 1996). In 1997, Zhuchenko *et al* analyzed the genotypes of a large ataxia patient group and 8 unrelated families, whom were found to contain moderate CAG expansions in CACNA1A (Zhuchenko, Bailey et al. 1997). Since 1997, more cases of SCA6 have been reported from Japan and German (Gomez, Thompson et al. 1997; Schols, Kruger et al. 1998).

Clinical manifestation and neuropathology

The clinical features of SCA6 include slowly progressive cerebellar ataxia, dysarthria, nystagmus (an involuntary, rapid, rhythmic movement of the eyeball) and vibratory sensory loss (Zhuchenko, Bailey et al. 1997). The age of onset varies from 20 to 66 years with an average of 45 years of age. SCA6 usually progresses over a 20 to 30 year period before its lethal end stage (Zhuchenko, Bailey et al. 1997). Neurophathologically, the cerebellar atrophy with the loss of Purkinje cells is severe, the brainstem shows mild atrophy, and moderate degeneration can be found in dentate nucleus and inferior olive. Both nuclear and cytoplasmic aggregates have been found in cerebellar Purkinje cells (Ishikawa, Owada et al. 2001).

Gene, mutation and protein

The CACNA1A gene is located on chromosome 19p13 and covers 300 kb with 47 exons. The CAG repeat lies in the coding region near the 3' end. The normal repeat size is 4-18 and in SCA6 the repeat length ranges from 21 to 33. It is an interesting exception among the polyglutamine diseases, with the threshold between normal and disease occurring in a repeat length range about one half that found in the other diseases (Bates 2002). CACNA1A gene codes for the alpha 1A subunit of voltage-gated calcium channels type P/Q, whose function is to control neurotransmitter release in synapses (Dunlap, Luebke et al. 1995). CACNA1A is expressed widely in the brain with the highest expression level in cerebellum (Fletcher, Lutz et al. 1996). Because of the important role of CANA1A in the neurons, the pathogenesis of SCA6 may be more complex than other polyglutamine diseases and the expansion of polyglutamine in the protein may gain more toxicity due to the location and the high local concentration, which may explain why the repeat length for disease onset is lower than other polyglutamine diseases (Zoghbi and Orr 2000).

Spinocerebellar Ataxia 7 (SCA7)

History of SCA7

SCA7 is distinguished from the other spinocerebellar ataxias due to the characteristic retinal degeneration that is associated with neurological dysfunction in this

disease (David, Durr et al. 1998). In 1995, the gene for SCA7 was mapped on chromosome 3p by linkage analysis (Holmberg, Johansson et al. 1995). By a novel technique called repeat expansion detection (RED), which detects repeat expansions directly from genomic DNA, the mutation of SCA7 was confirmed to be CAG repeat expansion in 1996 (Lindblad, Savontaus et al. 1996). In 1997, the SCA7 gene was discovered by positional cloning based on the previous information (David, Abbas et al. 1997).

Clinical manifestation and neuropathology

Clinically, SCA7 contains both the classic features of SCAs including ataxia, dysarthria, hyperreflexia (exaggeration of reflexes), and ophthalmoplegia (ocular muscular dysfunction due to the impairment of motor neurons that control the ocular muscles), as well as progressive vision defects, starting from blue-yellow dyschromatopsia (a condition in which the ability to perceive colors is abnormal) at the initial stages and evolving to complete blindness in the end (David, Durr et al. 1998). The age of onset of SCA7 varies from 1 to 70 years depending on the CAG repeat length with a mean at 29. The neuropathological features include atrophy in cerebellum and neuronal loss in inferior olive, dentate nucleus and cranial nerve nuclei. Retinal pathology includes loss of photoreceptors, ganglion and bipolar cells. SCA7 was reviewed in (Martin, Van Regemorter et al. 1999). Intranuclear intrabodies have been detected in several parts of the brain (Holmberg, Duyckaerts et al. 1998).

Gene, mutation and protein

The SCA7 gene is located on chromosome 3p12-13. The gene contains a 2727 bp open reading frame made up of 13 exons. The CAG repeat is located near the 5' end of the gene. The repeat length in unaffected individules is 4-35; it is 37-306 in the SCA7 patients (David, Abbas et al. 1997; Bates 2002). The gene encodes an 892 amino acid long (130kD) protein of unknown function, named ataxin-7 (David, Durr et al. 1998). Ataxin-7 is widely expressed in CNS and other tissue (Jonasson, Strom et al. 2002). It contains a nuclear localization signal (NLS), indicating a nuclear function of the protein. Ataxin-7 has also been found to be co-localized with an endoplasmic reticulum marker, BiP, a molecular chaperone, which functions to protect proteins from aggregation during assembly (Martin, Van Regemorter et al. 1999).

Spinocerebellar Ataxia 17 (SCA17)

History of SCA17

SCA 17 was discovered very recently. In 1999, Koide *et al* described (Koide, Kobayashi et al. 1999) a neurological disorder in a single Japanese individual caused by CAG expansion in the TATA-binding protein (TBP), raising the question "is this a new polyglutamine disease?". In 2001, six additional families (two German and four Japanese) were reported individually with CAG repeat expansion mutations in TBP associated with

neurodegeneration (Nakamura, Jeong et al. 2001; Zuhlke, Hellenbroich et al. 2001). Thus, SCA17 was discovered.

Clinical manifestation and neuropathology

The clinical features of SCA17 include ataxia, impaired pyramidal, extrapyramidal and autonomic systems, and severe intellectual impairment. The age of onset ranges from 19 to 48 years (Fujigasaki, Martin et al. 2001; Nakamura 2001). The postmortem brain tissue from one patient showed shrinkage and moderate loss of small neurons, with gliosis predominantly in the caudate nucleus and putamen and similar but moderate changes in the thalamus, frontal cortex, and temporal cortex. Moderate Purkinje cell loss and an increase of Bergmann glia were seen in the cerebellum (Fujigasaki, Martin et al. 2001). Immunocytochemical analysis performed with anti-ubiquitin and anti-TBP antibodies showed neuronal intranuclear inclusion bodies (Fujigasaki, Martin et al. 2001).

Gene, mutation and protein

TBP gene is located on chromosome 6q27. It normally contains a 25-44 CAG repeat, while in SCA17, it contains a 46-63 repeat (Fujigasaki, Martin et al. 2001; Nakamura 2001). The function of normal TBP is a DNA-binding subunit of RNA polymerase II transcription factor D (TFIID) (Peterson, Tanese et al. 1990), which is essential for the expression of almost all of the protein-encoding genes in the cells (Rowlands, Baumann et al. 1994).

Summary on the similarities and differences in polyglutamine diseases

The common features of the diseases are summarized as following:

1. Genetically the 9 diseases have a similar mutational basis: expansion of CAG repeats in the coding sequences. The repeat length cutoff for each of the disease is around 36-40 [except SCA6 (20) and SCA17 (46)] (Zoghbi and Orr 2000; Bates 2002). The diseases are mostly dominantly inherited (except for SBMA, explained in SBMA section). Other mutations in the same genes, such as missense mutations, nonsense mutations or completely depletion of the genes, which result in loss of the normal function of the gene products, cause different diseases, not polyglutamine diseases. For example, loss of AR gene result in testicular ferminization rather than neurodegeneration (SBMA) (Pinsky, Trifiro et al. 1992). Gene knockout mice models provide further evidence for the gain of a toxic function by the expanded polyglutamine sequence. However, recent studies have also suggested that at least in some cases there may be some combinations of gain of function along with the loss of the normal functions of the genes in the polyglutamine diseases (Lieberman and Fischbeck 2000; Cattaneo, Rigamonti et al. 2001).
2. The epidemiology of each disease reveals regional differences. This has been proposed to be due to the founder effect since many of the different families with certain polyglutamine diseases can be traced back to a common ancestor (Stevanin, Cassa et al. 1995; Wakisaka, Sasaki et al. 1995; Tanaka, Doyu et al. 1996; Didierjean, Cancel et al. 1999; Stevanin, David et al. 1999; Harper 2002).

3. Clinically, the diseases are exclusively neurodegenerative diseases: mid-life onset, progressive for about 10-30 years and ultimately fatal. Despite the wide expression of the mutated genes in most of the tissues in the body, neuronal system is the target for the diseases. The disease severity directly correlates with CAG repeat length, while the age of onset shows an inverse relationship with the repeat length. Massive expansions of polyglutamine result in severe cases of infantile and juvenile diseases with onset at very early ages, which progress very fast to death (Bates 2002).
4. From the neuropathological point of view, selected areas (with some overlap) of the brain in each disease show atrophy and neuronal loss as described above. The brain atrophy can be very dramatic in the late stage, due to massive neuronal loss. Again, the selective vulnerabilities of different subsets of the neurons in each disease are lost when massive CAG expansion occurs. The characteristic neuronal intranuclear inclusions (NII) are presented in most affected brains. Cytoplasmic aggregates have also been detected in HD, SCA2, and SCA6 (Bates 2002).
5. The gene products of the 9 polyglutamine disease are proteins with no homologies to each other except for their polyglutamine sequences. The functions of the proteins are largely unknown, except that AR and TBP are transcriptional factors and ataxin-6 is a calcium channel subunit. The cellular locations of the proteins can be either cytoplasmic or nuclear, while NII made of mutated proteins have been found in most of the diseases. The nine polyglutamine disease genes and gene products are compared and summarized in Tables 3 and 4.

Table 3. Summary on polyglutamine disease genes and gene products

Disease	Gene locus	Normal /expanded CAG repeat	Gene product	Protein size/ location of polyglutamine(aa#)	Molecular weight (kD)
HD	4p16.3	6-39/36-200	huntingtin	3144/18	348
SBMA	Xq11-12	9-33/38-65	AR	918/58	104
DRPLA	12p13.31	3-35/49-88	Atrophin1	1184/474	190
SCA1	6p23	6-44/39-83	Ataxin1	816/197	87
SCA2	12q24.1	13-33/32-200	Ataxin2	1313/166	145
SCA3	14q32.1	3-40/54-89	Ataxin3	359/292	46
SCA6	19p13.1	4-19/20-33	CACNA1A	2410/2328	280
SCA7	3p12-13	4-35/37-306	Ataxin1	893/30	95
SCA17	6q27	25-44/46-63	TBP	339/58	38

Table 4. Comparison of the cellular localizations of polyglutamine proteins and aggregates in different diseases and the involvement of proteolytic processing

Disease	Normal protein location	Aggregate location	Proteolytic cleavage
HD	cytoplasm	Nucleus & cytoplasm	yes
SBMA	nucleus	nucleus	yes
DRPLA	cytoplasm	nucleus	yes
SCA1	nucleus	nucleus	no
SCA2	cytoplasm	Nucleus & cytoplasm	yes
SCA3	cytoplasm	nucleus	no
SCA6	membrane	Nucleus & cytoplasm	unknown
SCA7	nuclear	nucleus	yes
SCA17	nucleus	nucleus	unknown

In summary, the common features presented by all of the polyglutamine diseases suggested a unified disease mechanism. This mechanism is centered in the expansion of polyglutamine tracts in the respective proteins in these different diseases.

Experimental model systems

Because the polyglutamine diseases are neurodegenerative diseases, it is impossible to get tissues directly from patients at an early stage of disease progression. Postmortem brain tissue represents the late stage of the disease pathogenesis and can only provide information that contains a mixture of early events and late events (that may be the results of an early event). This makes it particularly important to obtain model systems to facilitate research in discovering a cure. Indeed, a large variety of mouse, invertebrate, cellular and *in vitro* model systems have been established to study these diseases. Different model systems have both advantages and disadvantages. Sometimes they do not agree with each other on the very fundamental issues, like the toxicity of the polyglutamine aggregates. But the experimental models are invaluable in contributing to our understanding of the possible molecular and cellular mechanisms of the diseases. Here, I will briefly summarize the findings drawn from the experimental model systems with the focus on HD, since it the most intensively studied. The detailed mechanisms will be summarized in the section of molecular and cellular mechanism of the diseases.

Mouse models

The mouse model systems can be grouped into three different categories: (1) knock-out, which is the depletion of the mouse homolog of the gene; (2) transgenic model, which is expression of exogenous human polyglutamine containing gene, either full length or truncated; (3) knock-in, which uses gene-target strategies to replace the polyglutamine sequences in mouse homolog genes with the expanded version.

A mouse *huntingtin* homolog has been identified, named *Hdh*, which is 86% identical to the human at the DNA level and 91% identical at the protein level, but with only 7 polyglutamines compared to 6-36 polyglutamine in normal human (Barnes, Duyao et al. 1994). The extents of homology of all polyglutamine proteins in different organisms are summarized in Table 5. The homozygous *hdh* gene knock-out mouse is an embryonic lethal (Duyao, Auerbach et al. 1995), resulting in elevated apoptotic cell death (Zeitlin, Liu et al. 1995), which suggests that huntingtin is important for embryonic development and may be involved in an anti-apoptosis pathway. Furthermore, the heterozygous knock-out mouse is phenotypically normal, suggesting that the disease mechanism for Huntington's disease is most probably due to a gain of function, rather than a loss of function (Duyao, Auerbach et al. 1995). Transgenic mice with a fragmented human HD gene (often exon1) and an expanded CAG repeat show a progressive neurologic phenotype with many of the features of HD, including choreiform movements, tremor, seizures and weight loss. These mice usually die earlier than control mice. At the same time, the full-length human huntingtin transgenic mouse results in a less severe and later onset phenotype, suggesting that the cleavage of the full-length huntingtin is important

Table 5. The homology of polyglutamine proteins in different organisms at the protein level [a].

Diseases	Human	Mouse	Rat	Drosophila	C. elegant
HD	100%	91 %	90 %	-	-
SBMA	100%	84 %	85 %	25 %	26 %
DRPLA	100%	92 %	93 %	26 %	27%
SCA1	100%	85 %	87 %	23 %	39 %
SCA2	100%	87 %	25 %	20 %	21 %
SCA3	100%	26 %	85 %	-	33 %
SCA6	100%	92 %	91 %	59 %	61 %
SCA7	100%	21 %	-	-	26 %
SCA17	100%	98 %	40 %	62 %	55 %

[a] Numbers are obtained from NCBI website.

for pathogenesis (this will be explained later). The knock-in mice show only mild and very late onset phenotype in a long polyglutamine knock-in (150Q) model (Bates, Mangiarini et al. 1998).

Fewer mouse models have been generated for other polyglutamine diseases. Together with HD, the mouse models for different diseases are listed in Table 6. Taken together, the mouse model systems have successfully reproduced the late onset, progressive neurological phenotypes of polyglutamine diseases. Studies on these model systems shed light on the disease mechanisms. The findings include: (1) the instability of CAG repeat, both gametic and somatic (Bates and Davies 1997; Shelbourne, Killeen et al. 1999); (2) the disease is mainly due to a gain of toxic function by expanded polyglutamine protein, but also contains some of degree of loss of normal function of the proteins due to the expansion of polyglutamine (Duyao, Auerbach et al. 1995); (3) the processing of the full length protein is the first step of the pathogenesis and very important for the region selectivity of different diseases (Bates, Mangiarini et al. 1998); (4) neuronal dysfunction, including mitochondrial, presynaptic and synaptic disruption, occurs at a very early stage, which may account for the early symptoms of the polyglutamine diseases, and then progresses to neuronal death (Hodgson, Agopyan et al. 1999); (5) Gene expression pattern changes have been revealed by gene expression microarray studies (Luthi-Carter, Strand et al. 2000); (6) Polyglutamine aggregate pathology and neurodegeneration have been described in virtually all of the mouse models (Bates 2002), indicating its importance to the disease progression. However, there is controversy regarding a direct toxic role of polyglutamine aggregates and the

Table 6. Mouse models for polyglutamine diseases

Disease	Model design	reference
HD	Knock-out 1	(Duyao, Auerbach et al. 1995)
	Knock-out 2	(Zeitlin, Liu et al. 1995)
	Transgenic 1, with human HD exon 1 (1-90) under human HD promoter (20Q,115Q and 150Q)	(Mangiarini, Sathasivam et al. 1996)
	Transgenic 2, with human HD 1-171 under prion promoter (18Q, 44Q and 82Q)	(Schilling, Becher et al. 1999)
	Transgenic 3, with human HD 1-1000 under Rat NSE promoter (10Q, 46Q and 102Q)	(Laforet, Sapp et al. 2001)
	Transgenic 4, with human HD full length cDNA under CMV promoter (18Q, 50Q and 91Q)	(Reddy, Williams et al. 1998)
	Transgenic 5, with yeast artificial chromosome (YAC)containing original human full length HD gene (18Q, 46Q and 72Q)	(Hodgson, Agopyan et al. 1999)
	Transgenic 6, with HD exon 1 under an inducible tetO/CMV promoter (94Q)	(Yamamoto, Lucas et al. 2000)
	Knock-in 1 (72Q and 80Q)	(Shelbourne, Killeen et al. 1999)
	Knock-in 2 (20Q, 50Q, 92Q and 111Q)	(Wheeler, White et al. 2000)
	Knock-in 3 (73Q and 96Q)	(Levine, Klapstein et al. 1999)
	Knock-in 4 (80Q and 150Q)	(Lin, Tallaksen-Greene et al. 2001)

Table 6 continued.

Disease	Model design	Reference
SBMA	Transgenic 1, with truncated AR under the promoter of prion or NFL (112Q)	(Abel, Walcott et al. 2001)
	Transgenic 2, with 239Q under human AR promoter	(Adachi, Kume et al. 2001)
DRPLA	Transgenic model with full length human atrophin1 under prion promoter (65Q)	(Schilling, Wood et al. 1999)
SCA1	Transgenic 1, with human ataxin1 under PcP2 promoter (82Q)	(Burright, Clark et al. 1995)
	Transgenic 2, modified from transgenic 1 with mutated NLS	(Klement, Skinner et al. 1998)
	Knock-in with mouse ataxin1 (78Q)	(Lorenzetti, Watase et al. 2000)
SCA2	Transgenic, with human ataxin 2 under pcp2 promoter (58Q)	(Huynh, Del Bigio et al. 1999)
SCA3	Transgenic with truncated human ataxin 3 under pcp2 promoter (79Q)	(Ikeda, Yamaguchi et al. 1996)
SCA7	Transgenic 1, with human ataxin 7 under pcp2 promoter (90Q)	(Yvert, Lindenberg et al. 2000)
	Transgenic 2, with human ataxin 7 under PDGF-B promoter (128Q)	(Yvert, Lindenberg et al. 2001)
	Transgenic 3, with human ataxin 7 under prion promoter (92Q)	(La Spada, Fu et al. 2001)

mechanisms involved in this toxicity (this will be explored further in the mechanism section).

Drosophila and C elegans models (Invertebrate models)

Drosophila has also been used as a model system for some of the polyglutamine diseases, including HD (Jackson, Salecker et al. 1998), SCA1 (Fernandez-Funez, Nino-Rosales et al. 2000), SCA3 (Warrick, Paulson et al. 1998) and all polyglutamine diseases using a pure polyglutamine sequence (Kazemi-Esfarjani and Benzer 2000; Marsh, Walker et al. 2000). The disease phenotypes vary from disruptions of the eye retina (photoreceptors) to shortening of the life span of the flies. Drosophila models can not only contribute to a better understanding of the disease mechanism, but also serve as an *in vivo* screening tool to discover possible modifiers for the toxicity of polyglutamine proteins. Indeed, heat shock protein (Fernandez-Funez, Nino-Rosales et al. 2000) and histone deacetylase (HDAC) inhibitors (Steffan, Bodai et al. 2001) have been shown to rescue the disease phenotype in drosophila models, suggesting that both protein misfolding and transcriptional dysregulation are involved in the disease mechanisms. Strategies involving up-regulation of the heat shock response and use of HDAC inhibitors may lead to therapeutic approaches for polyglutamine diseases.

Studies using C. elegans as a model system have been carried out using truncated huntingtin (Faber, Alter et al. 1999; Parker, Connolly et al. 2001) and GFP tagged polyglutamine (Satyal, Schmidt et al. 2000). The abnormal phenotypes were measured by a touch response defect (suggesting the neuronal dysfunction), growth retardation and

neuronal cell death. The neuronal cell death in the model requires *ced-3* (caspase homolog) activation, which suggests that cell death is apoptotic (Faber, Alter et al. 1999).

Cellular models

The cellular mechanisms of polyglutamine diseases have been explored by the generation and study of a variety of transfected cell models for polyglutamine diseases. By transiently/stably overexpressing the full length/truncated huntingtin (Lunkes and Mandel 1998; Martindale, Hackam et al. 1998), AR (Butler, Leigh et al. 1998), atrophin-1 (Igarashi, Koide et al. 1998), ataxin1 (Skinner, Koshy et al. 1997) and ataxin-3 (Ikeda, Yamaguchi et al. 1996; Paulson, Perez et al. 1997) in different cell lines, the cell models suggest a polyglutamine length dependent toxicity effect. The toxicity requires nuclear localization and activation of caspase. Upregulation of heat shock protein can decrease the severity of the toxcity (Sittler, Lurz et al. 2001; Waelter, Boeddrich et al. 2001). Truncated forms and NLS targeted polyglutamine proteins are much more toxic than full length and NES targeted ones (Saudou, Finkbeiner et al. 1998; Perez, Paulson et al. 1999; Yasuda, Inoue et al. 1999). The source of cell specificity is obscure, since cell models build in a monkey fibroblast cell line (Cos-7 cells) also show similar effects (Skinner, Koshy et al. 1997). This may be due to overexpression of polyglutamine proteins in these models.

In vitro models

In vitro models of interest are studies focused on the biochemical and biophysical features of polyglutamine protein, including aggregation kinetics and the structures of the

aggregates. These studies include using either recombinant polyglutamine protein (Scherzinger, Lurz et al. 1997; Scherzinger, Sittler et al. 1999) or chemically synthesized polyglutamine peptides (Berthelier, Hamilton et al. 2001; Chen, Berthelier et al. 2001; Chen and Wetzel 2001; Chen, Berthelier et al. 2002; Chen, Ferrone et al. 2002) to carry out the protein aggregation in the test tube. By using various techniques of measuring the staining features of the aggregates, the aggregation kinetics and the recruitment kinetics of polyglutamine aggregates, these studies have revealed that the polyglutamine protein can self-assemble into aggregates and this process is a nucleation-dependent polymerization (Scherzinger, Sittler et al. 1999; Ross, Poirier et al. 2003). The polyglutamine aggregates possess an amyloid-like fibril structure (Chen, Berthelier et al. 2002). A detailed explanation of the protein aggregation kinetics and the structure of the polyglutamine aggregates are illustrated in the molecular and cellular mechanisms section. Studies of inhibitors for polyglutamine aggregation or elongation may potentially lead to drugs for curing the polyglutamine diseases.

Molecular and Cellular Mechanisms of polyglutamine diseases

Polyglutamine sequences

The glutamine repeat has been found to be the most common amino acid repeat in the known protein database (Green 1998). The functions of the polyglutamine sequences in each protein are not clear. A BLAST search (provided by NCBI) of the swissprot protein database reveals that about 50% of the polyglutamine containing proteins are

transcription factors, or nuclear proteins that may function in gene transcription, and that these represent about 2% of the total nuclear protein (data not shown). This suggests that the polyglutamine sequence may be involved in gene transcription. Indeed, *in vitro* studies have shown that a polyglutamine sequence can activate gene transcription when fused to a DNA binding protein (Gerber, Seipel et al. 1994). It is still not clear how polyglutamine protein activates gene transcription - by directly interacting with the DNA or by interacting with other transcription factor proteins. Polyglutamine sequences seem to be particularly long in the proteins of primates, compared to those of other species. The role these sequences may play in evolution is still very obscure (Green 1998). In any event, the particularly long polyglutamine sequences in primates and their tendency to expand, serve as the basis for the unique problem of CAG repeat expansion that human beings must deal with.

Polyglutamine expansion and protein misfolding

None of the structures of the 9 disease-related polyglutamine containing proteins, either normal or expanded are known. However, the expansion of the polyglutamine sequence may result in the protein misfolding. Protein chaperones and the components of the ubiquitin-proteasome system have often been found colocalized with polyglutamine aggregates in the postmortem brain tissue of polyglutamine diseases, transgenic mice models and transfected mammalian cell lines (Cummings, Mancini et al. 1998; Chai, Koppenhafer et al. 1999; Waelter, Boeddrich et al. 2001). Protein chaperones usually function in assisting protein folding and correcting protein misfolding and the UPS system (to be explained in detail later) is the key pathway for degradation of the

misfolded proteins if they fail to be corrected by chaperones. The colocalization of these components with protein aggregates suggests their involvement in the processing of misfolded protein. Moreover, overexpression of chaperone proteins together with the expanded polyglutamine proteins has been shown to decrease the aggregation and toxicity of polyglutamine proteins in transgenic mice (Cummings, Mancini et al. 1998), drosophila (Warrick, Chan et al. 1999) and transfected cell models (Krobitsch and Lindquist 2000). In polyglutamine transfected mammalian cell model systems, studies using the drug Geldanamycin, which can stimulate the heat shock response in cells and therefore induce expression of chaperones, can also decrease mutant huntingtin protein aggregation (Sittler, Lurz et al. 2001). This discovery indicates a possible therapeutic approach for polyglutamine diseases.

Protein misfolding may result in (a) the exposure of the cleavage site and make it accessible for protease digestion, which then may process the full length protein into a more toxic and highly aggregation-prone fragment containing the expanded polyglutamine tract; (b) protein aggregation, which leads to subsequent cellular dysfunction and cell death, as further discussed below.

Cleavage of the full length protein

The cleavage of full length mutant protein has been demostrated in HD, SBMA, DRPLA, SCA2 and SCA7, but not in SCA1 and SCA3 (Bates 2002), as indicated in Table 4. Studies of SCA6 and SCA17 on this aspect have not been reported. Transgenic mice expressing the N-terminal fragment of huntingtin generate more severe, earlier onset phenotypes compared to knock-in mice (full length mouse huntingtin with an

expanded polyglutamine sequence) or mice expressing full length mutant human huntingtin. The processing of polyglutamine containing proteins may contribute to further pathogenesis of the diseases through three possible mechanisms. First, it may facilitate polyglutamine protein interaction and further aggregation by eliminating other amino acids that may prevent aggregation. Evidence supporting this comment includes the observation that the recombinant glutathione S-transferase (GST) tagged huntingtin exon1 protein is soluble *in vitro* until the cleavage and removal of GST, which then results in polyglutamine aggregation (Scherzinger, Lurz et al. 1997). Second, the cleaved fragment containing the expanded polyglutamine sequence is often found relocalized into the nucleus (Li, Nakagomi et al. 1998). This may be due to a smaller size that may facilitate nuclear entry, or the elimination of a nuclear export signal (NES) that lies in the other part of the protein (Schilling, Wood et al. 1999). The NES usually dominates over an NLS in determining the cellular location of the protein (Schilling, Wood et al. 1999). The translocation of expanded polyglutamine protein into the nucleus appears to be very important for the disease pathogenesis as will be explained later in this chapter. Third, studies have shown that the expanded polyglutamine protein is cleaved by caspases 3 and 6 (Wellington, Ellerby et al. 1998; Wellington, Singaraja et al. 2000). The activated caspases can then lead to apoptosis and neuronal loss. The initial activity of the caspases for cleavage of polyglutamine proteins may be from the low level intrinsic zymogen before they are cleaved into the highly active form. Another hypothesis is that the expanded polyglutamine containing protein may cause subtle cellular dysfunction before being cleaved, which may result in the activation of a small amount of caspase 3 and 6

(Wellington and Hayden 2000). This event may be slow at the beginning, but might be amplified by the ability of the toxic fragment to generate more caspase activation through increased cellular dysfunction, which then cleave even more toxic fragments, and this can go into a cycle which amplifies the toxic effect (Wellington and Hayden 2000). The caspase inhibitor (z-VAD-fmk) has been shown to increase the lifetime of the HD transgenic mice, suggesting a possible therapeutic approach (Ona, Li et al. 1999).

Polyglutamine aggregation

A large amount of data from clinical studies, animal models and invitro models has suggested that polyglutamine aggregation is the center for the disease mechanism. But how are these aggregates formed?

An early hypothesis by Green *et al* in 1993 suggested that the aggregation of polyglutamine proteins might be mediated by transglutaminase (TG) cross-linking (Green 1993). TG can catalyze the reaction forming a covalent glutamyl-lysine isopeptide bond between the two substrates in the presence of calcium (Greenberg, Birckbichler et al. 1991). This results in the cross-linking of polyglutamine proteins and further facilitates their aggregation. This hypothesis was supported by an *in vitro* study showing that expanded polyglutamine containing protein is a good substrate for TG (Kahlem, Terre et al. 1996). However, the hypothesis is not supported by evidence from a recent study, in which repression of TG expression, by an antisense TG mRNA, failed to decrease the aggregation and cytotoxicity of a truncated huntingtin fragment with expanded polyglutamine (Chun, Lesort et al. 2001).

Another hypothesis that has been generally accepted, called "polar zipper" formation, was proposed by Max Perutz (Perutz 1996): two antiparallel β-strands of polyglutamine repeats can be linked together by hydrogen bonds between the main-chain and side-chain amides and undergo polymerization and this will ultimately lead to aggregation by "polar zipper" formation and the formation of amyloid-like aggregates. This hypothesis has been greatly supported by the study using bacterial recombinant N-terminal fragment of huntingtin that can self aggregate into amyloid-like structures in a concentration and polyglutamine length dependent manner (Scherzinger, Lurz et al. 1997; Scherzinger, Sittler et al. 1999). This suggested that the aggregation of polyglutamine proteins is a self-assembly process.

Studies on the aggregation kinetics by various labs, including our lab, revealed a nucleation dependent fibrillogenesis of polyglutamine peptides, which is similar to the Aβ amyloid formation in Alzheimer's disease (AD) (Chen, Berthelier et al. 2002; Wanker 2002; Ross, Poirier et al. 2003). The first phase of the aggregation kinetics is a slow nucleation phase as the polyglutamine protein has to adopt conformational changes to form a nucleus. Studies using chemically synthesized peptides (Altschuler, Hud et al. 1997; Chen, Berthelier et al. 2001) suggest that the monomeric form of polyglutamine peptides, both short and long, adopt mainly random coil structure, in contrast to the β-sheet rich structure of the aggregated from (Chen, Berthelier et al. 2001). A study in our lab suggested that the nucleus is a single polyglutamine peptide rather than an oligomeric nucleus as is typically visualized for amyloid assembly (Chen, Ferrone et al. 2002). The second phase is the fast growth phase when additional polyglutamine monomers

associate with the nucleus and form large fibrils. The last phase is when no more net growth occurs, which is when the reaction reaches its equilibrium. The critical concentration (the lowest concentration before polyglutamine peptides start to aggregate) and lag time (the shortest time before the peptides start to aggregate) determine the occurrence of the aggregation. In polyglutamine proteins, the longer the polyglutamine length, the shorter the lag phase and the lower the critical concentration needed for aggregation. This may explain why expanded polyglutamine proteins cause disease, and may also explain the correlation between the age-of-onset of the disease and the polyglutamine length (Chen, Ferrone et al. 2002). Studies also suggest that the elongation of polyglutamine aggregates fits into a docking-locking-blocking model (Berthelier, Hamilton et al. 2001), in which a polyglutamine monomer recognizes the binding site of a fibril and binds to it (docking; this step is reversible), after which the bound monomer undergoes a conformational change (locking; this step is irreversible) and generates a new binding site for accepting another monomer. Another monomer then binds to this new site and inhibits the dissociation of the previous bound peptide (blocking).

Polyglutamine aggregates observed in the NIIs in postmortem HD brain tissue (Huang, Faber et al. 1998; McGowan, van Roon-Mom et al. 2000), extracted aggregates from transfected cell models (Scherzinger, Lurz et al. 1997; Scherzinger, Sittler et al. 1999) and the *in vitro* self-assembly polyglutamine aggregates (Chen, Berthelier et al. 2002) exhibit some, but not all of the features of a typical amyloid, which made them amyloid-like aggregates. These features include the fibrillar ultrastructure that is often obtained by EM and Thioflavin T (ThT) binding-dependent fluorescence (which was

used in our lab as an indicator for aggregate formation). Congo red staining and exhibiting green birefringence under polarized light (a typical feature for amyloid) has been reported. However, only limited number of studies have shown this, most of which are just the staining of the polyglutamine aggregates by Congo red.

In regards to the toxicity of polyglutamine aggregates, the issue is still under debate. Given the fact that NII is a hallmark of the diseases and that the affected brain regions correlate well with the formation of the aggregates, aggregates seem to be closely associated with the disease. The presence of polyglutamine aggregate pathology in virtually all of the transgenic mice models is also supportive of the possible toxicity of the polyglutamine aggregates. A cellular study with over-expression of the expanded polyglutamine containing proteins results in cell death (Ikeda, Yamaguchi et al. 1996), which further supportes a possible toxic role of polyglutamine aggregates. On the other hand, a direct toxic role of the polyglutamine aggregates has been questioned and aggregates were hypothesized as "by-product" or even "protective" for the neurons, based on the evidence that (a) the postmortem brain specimen showed NII in healthy neurons as well; (b) some HD transgenic mouse models showed NII formation that is not associated with cell death (Davies, Turmaine et al. 1997); (c) in a transgenic SCA1 mouse study, the self association domain (not polyglutamine sequence) of ataxin 1 was depleted (Klement, Skinner et al. 1998), which resulted in no visible protein aggregation in the mouse, but the mouse still develop neurodegeneration. Also, as the process of forming the aggregates from monomer occurs in the cells, there will be monomer, intermediate and mature aggregates present in the cells. Which species is/are the toxic

species? The current data using a transfected model cannot directly answer these questions and the role of aggregates in cellular toxicity is the subject of the present work.

In regards to the current popular topic on the intermediate toxicity in Aβ and other aggregated proteins (Bucciantini, Giannoni et al. 2002), there are no reports in the literature directly addressing the toxicity of polyglutamine intermediates. In addition, the intermediate toxic effect is mainly observed as extracellular toxicity (in Alzheimer's disease) rather than intracellular (in polyglutamine diseases).

Nuclear localization and transcription dysregulation

Regardless of the original location of the normal polyglutamine containing proteins, the aggregated mutant proteins have been found relocated to the nucleus of affected neurons, as shown in Table 4. The mechanism of how the aggregates are relocated to the nucleus is not clear. It may be mediated through a putative NLS or the aggregates may be carried in by interacting with other polyglutamine containing nuclear proteins (Bates 2002). But at what stage, monomeric or aggregated, the aggregates get into the nucleus, is unknown. Nonetheless, several studies have pointed out that the nuclear localization is very important in the disease pathogenesis. When a nuclear localization signal (NLS) in the expanded polyglutamine containing protein was mutated in an SCA1 transgenic mice model, the mice did not develop neurodegenerative disease (Klement, Skinner et al. 1998). Using the exogenous NES on the huntingtin construct, which excludes the hutingtin protein from the nucleus, dramatically reduces cellular toxicity (Saudou, Finkbeiner et al. 1998; Peters, Nucifora et al. 1999).

The importance of nuclear localization of the polyglutamine aggregates suggested that the aggregates may interrupt some nuclear function. And indeed, a large amount of studies have suggested dysregulation of gene transcription in polyglutamine expansion disease models. DNA microarray studies on gene transcription have revealed an abnormal gene transcription pattern in HD transgenic mice (Luthi-Carter, Strand et al. 2000). The abnormal gene transcription is mainly in the neurotransmitter receptors, intracellular signaling mechanisms, retinoic acid receptor machinery and calcium homeostasis system (Cha 2000; Luthi-Carter, Strand et al. 2000). CREB-binding protein (CBP) (Kazantsev, Preisinger et al. 1999; McCampbell, Taylor et al. 2000; Nucifora, Sasaki et al. 2001) and other transcription factors including TBP (Shimohata, Onodera et al. 2000) and Sp1 (Perez, Paulson et al. 1998) have been shown to be recruited into polyglutamine aggregates and sequestered. In support of the CBP study, a disease caused by loss of CBP is also neurodegenerative (Murata, Kurokawa et al. 2001). Recent studies show that histone deacetylase (HDAC) inhibitors rescue the neurodegeneration caused by polyglutamine diseases in drosophila (Steffan, Bodai et al. 2001). The mechanisms by which polyglutamine cause the dysregulation of gene transcription may be via interactions with transcriptional factors mediated by polyglutamine recruitment (recruitment sequestration mechanism) (Ross 2002).

Ubiquitin proteasome system (UPS) disruption

The UPS is an important proteolytic pathway in cells in addition to the lysosome and calcium dependent (capain) pathways. It is responsible for up to 90% of short-lived protein turnover, and plays an important role in maintaining normal cellular functions

(Mayer 2000; Vu and Sakamoto 2000). The ubiquitin-proteasome pathway involves two steps: the ubiquination step and the proteasome degradation step. First, the small ubiquitin protein (76aa) is activated by a ubiquitin –activating enzyme (E1), then transferred to a ubiquitin-conjugating enzyme (E2) and finally ligated to the protein substrate by a specific ubiquitin-protein ligase (E3), leading to the formation of an isopeptide bond between the C terminus of ubiquitin (Gly 76) and the side chain of a lysine residue on the protein substrate. By repeating this same reaction, a polyubiquitin chain can be formed to amplify the signal. Second, the ubiquinated protein is directed to the 26S proteasome, which is a multicomponent protease complex made of two 19S caps and one 20S core. The protein is then degraded in the proteasome into polypeptides or single amino acids (Schwartz and Ciechanover 1999). In polyglutamine disease, a large number of components of the UPS, including ubiquitin, some E2, E3 and 20S proteasome components, are found to be trapped into the aggregates (Davies, Turmaine et al. 1997; DiFiglia, Sapp et al. 1997). These findings indicated that (a) the UPS is involved in degradation of the misfolded protein containing the expanded polyglutamine tracts and (b) the UPS fails to degrade at least some expanded polyglutamine proteins, instead becoming trapped into protein aggregates, resulting in loss of normal UPS function. This may in turn result in abnormal accumulation of other proteins destined for degradation, which further results in cell dysfunction and cell death (Bence, Sampat et al. 2001). Treating cells with a proteasome inhibitor, lactacystin, can lead to an increased polyglutamine aggregation and toxicity in a transfected cell model (Cummings, Reinstein et al. 1999). This further supports the importance of the UPS in polyglutamine diseases.

Neuronal cell death

Undoubtedly, the dramatic brain atrophy observed in postmortem brain tissue results from severe neuronal cell death occurring in the late stages of polyglutamine disease (DiFiglia, Sapp et al. 1997). This was further confirmed by various animal and cellular model studies (Evert, Wullner et al. 2000). But the mechanism of the cell death remains to be determined.

Cell death mechanisms are divided into two based categories, apoptosis and necrosis, by their distinct features (Kerr, Wyllie et al. 1972). Apoptosis refers to an "inherently programmed phenomenon" and necrosis refers to a "violent and rapid" disruption of major cell functions. The detailed key features of apoptosis and necrosis are summarized and compared in Table 7. However, recent studies suggest that there are other cell death mechanisms that contain some of the key features of either or both these two types of cell death, but which cannot fit exactly into either of the categories (Bursch, Ellinger et al. 2000). A broader grouping of cell death mechanisms separates cell death into two classes: programmed or non-programmed cell death. Programmed cell death (PCD) is defined as requiring new gene activation and protein synthesis to mediate cell suicide. This class of cell death includes, in addition to apoptosis, autophagic PCD (a unique cell death type induced by certain DNA damage, that requires gene transcription, but dose not follow the caspase cascade, and instead proceeds through autophagic vacuoles formation and self-destructure (Bursch, Ellinger et al. 2000)), paraptosis (a newly proposed cell death type that also requires new protein synthesis, but presents a necrotic-like morphologic change (Sperandio, de Belle et al. 2000)) and perhaps others

Table 7. Summary and comparison of apoptosis and necrosis

Features	Apoptosis	Necrosis
Inducers	Developmental/Programmed Degenerative changes Growth factor depletion Mild ischemia, radiation, etc.	Severe and sudden injury, physical or chemical trauma (toxic, massive ischemia and high dose radiation, etc.)
Cellular consequences	1st phase: chromatin condensation; cell shrinkage (expose of phosphatidylserine on the membrane is a very early phenomenon) 2nd phase: fragmentation of both nucleus and cytoplasm and form apoptotic body 3rd phase: degradation of residual nuclear and cytoplasmic structures in phagosome, very little or no inflammation.	1: Cytoplasmic swelling 2: Rapid plasma membrane rupture 3: Organelles breakdown with remarkably little nuclear change 4: Nuclear swelling and break-down and development of massive inflammation.
Nuclear changes	1: Chromatin condensation and large fragmentation 2: DNA laddering: 180-200bp DNA fragmentation	1: Plumping and lysis of nucleus. 2: Diffuse and random DNA degradation
Cytoplsmic changes	1: Alterations of cytoskeleton resulting in rapid budding and blebbing of the plasma membrane. 2: Involution and contraction of cell organelles (Mitochondria may release cyto c, but not very obvious swelling, still maintain the contact structure.) 3: Cells lose contact with other cells and round up or float in the medium in cell culture.	1: ER swelling, vesiculation and rupture of membranes 2: Mitochondria: swelling and lose cristae, misshappen and rupture and lose intramitochondrial granules. 3: Lysosome: swells and rupture

yet to be defined. Non-programmed cell death is often used synonymously with necrosis, since its definition is broad enough to cover all the features described for a non-programmed cell death. In the following paragraph, I will focus mainly on apoptosis mechanistic pathways.

Studies on apoptosis signal pathways suggest that apoptosis mainly involves two pathways, which are dependent on the stimuli or inducers: a death receptor pathway and a mitochondrial pathway (Strasser, O'Connor et al. 2000).

The death receptor pathway can be induced by a variety of extra cellular stimuli, including cytokines like TNFα (tumor necrosis factor α) and Fas-L (Fas ligand). These death ligands can bind to the death receptor TNFR-1 and Fas/CD95/Apo-1 and cause trimerization and activation of the receptor. The activation of the receptors results in a conformational change and exposure of the intracellular death domain (DD), which then recruits a DD-containing adaptor protein such as FADD (Fas associated death domain protein). FADD then undergoes oligomerization, exposing its DED domain (death effector domain), which in turn recruits DED domain-containing initiator procaspases (8, 10). Procaspase 8 and 10 also undergo oligomerization and then auto-cleave or inter-cleave between each other to generate activated forms of caspases. Caspase 8 and 10 can directly cleave and activate "effector" caspases (3, 6, 7) (Earnshaw, Martins et al. 1999), which then cleave their substrates, leading to the apoptosis phenomenon: (a) cleavage of cytoskeleton proteins like actin, gelsolin, etc., resulting in cytosleleton rearrangement and membrane blebbing; (b) cleavage of nuclear structural proteins like lamin, nuclear matrix protein, etc., resulting in nuclear shape changes; (c) generation of the 180-200bp

internucleosomal DNA fragments, which is a characteristic feature detected on agarose gel, called the DNA ladder. This ladder results from cleavage of DFF (DNA fragmentation factor) 45/40, and release of DFF 40 which can oligomerize into large Dnase, cleaving double stranded DNA at internucleosome positions; (d) among the caspase substrates, Poly (ADP-ribose) polymerase (PARP) can be cleaved to an 85kD fragment, and this has been used as a method to detect apoptosis.

The mitochondrial pathway (Desagher and Martinou 2000) is usually death receptor independent and intracellularly induced. For example, growth factor withdrawal and DNA damage can activate the mitochondrial pathway for apoptosis, which is mediated through the proapoptotic BCL-2 family, proteins that can disrupt mitochondrial integrity and induce release of cytochrome c. On its release cytochrome c (a 13 kD electron transfer protein) binds to adaptor protein Apaf-1 in an ATP/dATP dependent manner and causes oligomerization of Apaf-1, which in turn recruits procaspase 9. On the recruitment to Apaf-1, procaspase 9 also undergoes oligomerization, which leads to its cleavage to an active caspase 9. Caspase 9 can further recruit and activate downstream effector caspases 3, 6, 7, and result in activating of apoptosis cascade (Earnshaw, Martins et al. 1999). The apoptosis pathway is illustrated in Figure 5.

As for polyglutamine diseases, although thzxe exact pathway for cell death is not completely understood, there is evidence suggesting an apoptotic cell death mechanism. DNA fragmentation has been detected by TUNEL staining in both HD brain (Dragunow, Faull et al. 1995) and a mouse model (Reddy, Williams et al. 1998). Caspase activation has been detected (Sanchez, Xu et al. 1999), and inhibition of caspase activation by Z-

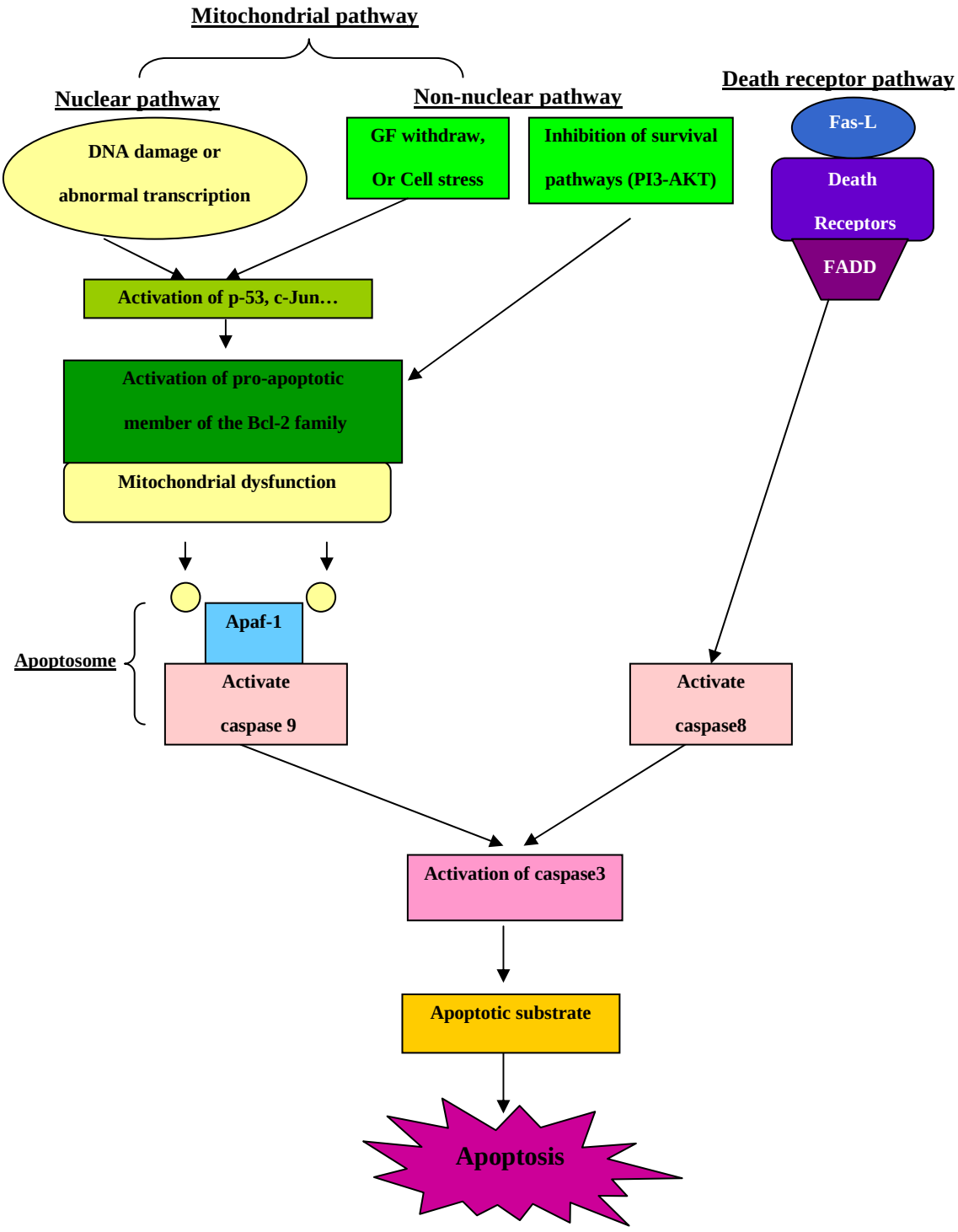


Figure 5. General cell signaling pathways for apoptosis

VAD-FMK can increase cell survival (Kim, Lee et al. 1999) (Ona, Li et al. 1999). On the other hand, the presence of gliosis in the affected brain in some polyglutamine diseases suggests the involvement of necrosis. Nonetheless, the bulk of the data gathered so far suggests a nonclassic apoptosis pathway for polyglutamine disease (Gutekunst, Norflus et al. 2002).

Neuropil aggregates and neuron projection failure

There is an emerging hypothesis on the mechanism of the polyglutamine diseases, especially on HD, which suggests that the dysfunction of neuronal synaptic transportation, due to its physical blockage by aggregates in the dendrites, or due to the degeneration of the dendrites induced by the toxic effect of the aggregates, may be responsible for the death of projected neurons due to lack of support (Sapp, Penney et al. 1999). In HD, this hypothesis was supported by the finding of the accumulation of the aggregates in the dendrites of corticostriatal projection neurons (Gutekunst, Norflus et al. 2002), which may be responsible for neuronal death of the striatal neurons. In another study on the ataxin-7 transgenic mouse model, in which the expression of ataxin-7 was controlled by a specific promoter that restricts the expression of ataxin-7 to certain designated neurons, neuronal degeneration occurs postsynaptic to the neurons expressing ataxin-7 and containing ataxin7 aggregates (Yvert, Lindenberg et al. 2000), suggesting a neuronal projection failure mechanism.

Cell specificity

Despite the wide expression of polyglutamine proteins in a variety of tissues in the body, and their diverse functions, only neurons appear to be victimized by polyglutamine expansion disease. The source of this cell type specificity remains unknown. Possible hypothesis have been made that if the polyglutamine aggregation is the basis for the disease mechanism, the critical concentration for aggregation (Chen, Ferrone et al. 2002) can only be reached in the cells that are long lasting, such as neurons (Ferro, dell'Eva et al. 2001).

As for the neuronal specificity observed in each polyglutamine diseases, there are a number of possible explanations. (1) The expression levels of the polyglutamine proteins for different cell types may be different for each disease, which would be expected to result in the protein aggregation kinetics also being different, since the aggregation process is concentration-dependent (Kosinski, Cha et al. 1997); (2) Differing functions of the different disease proteins, and /or differing profiles of interacting proteins (e.g., in a recruitment mechanism) for each disease proteins, may also account for the specificity in each disease; (3) The protease that processes the expanded polyglutamine proteins, the chaperones that protect the proteins against misfolding, and the ubiquitin-proteasome system that facilitates the degradation of incorrect proteins may vary in different neurons, which could likewise result in different responses to polyglutamine expansion and aggregation in different neurons; (4) The domain structure of different polyglutamine containing proteins have no relationship to each other except for the polyglutamine sequence itself; these unshared domains may specify each disease

with its special features, as an overlay above the shared polyglutamine aggregation driving forces of the pathology. In support of this, fragmented polyglutamine disease proteins seem to cause a more widespread neurodegenerative disease, with loss of regional specificity (Bates 2002).

The possible cell death mechanisms are illustrated in Figure 6.

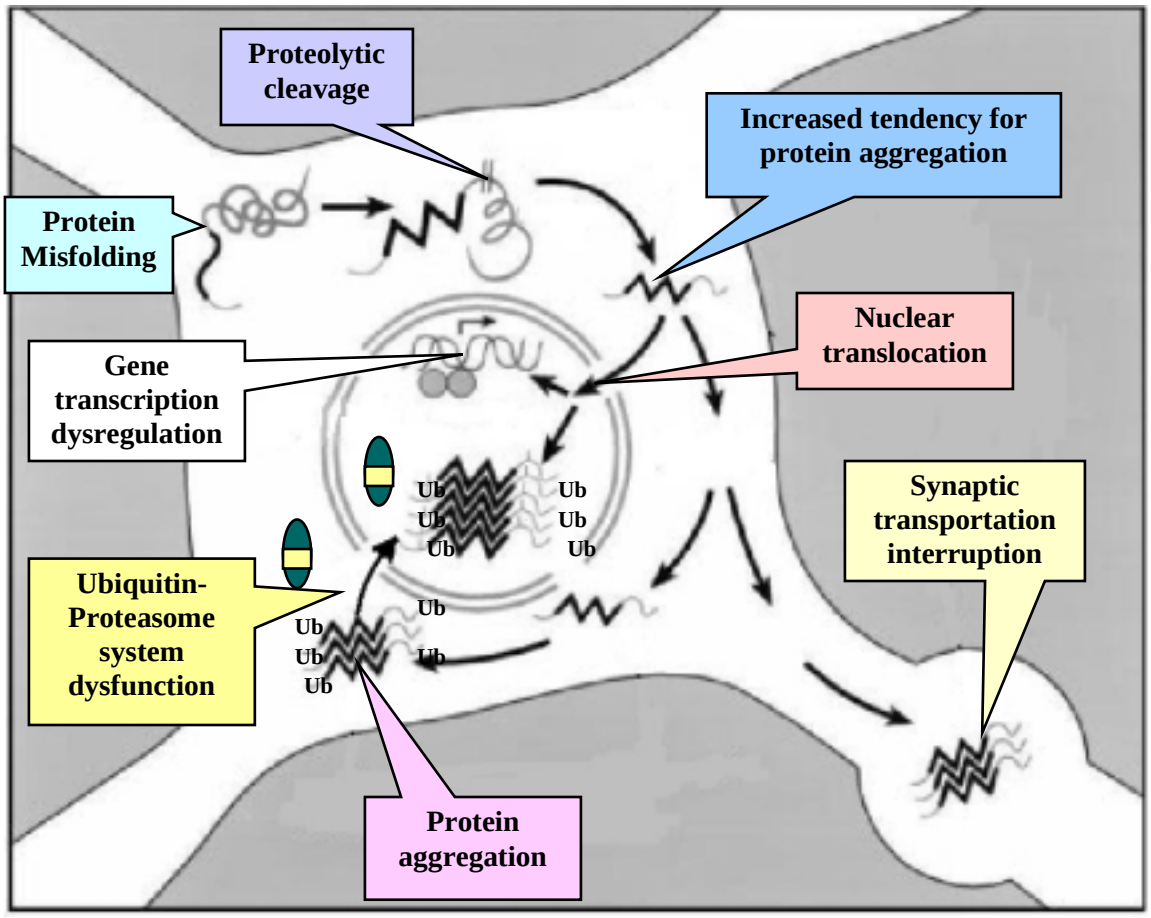


Figure 6. Summary on the hypothesis of the cellular mechanism of polyglutamine disease. Modified from (Ross, Wood et al. 1999)

CHAPTER 2

INTRODUCTION AND RATIONALE TO PRESENT WORK

In the polyglutamine diseases, evidence drawn from extensive studies has led to theories on the molecular and cellular mechanism of the disease that focus on a long polyglutamine sequence in the respective proteins，as encoded by an expanded CAG repeat in the open reading frame of the affected genes (reviewed in chapter1). But how these expanded polyglutamine sequences are toxic to neurons is not clear. A number of model systems based on expression of the mutant gene have been generated in order to understand the mechanisms of the disease and to discover a possible cure. However, there are still questions that cannot be directly answered by these model systems, and ambiguities that cannot be explained by these studies. Among these are: a direct toxic role of the disease pathologic hallmark, normally, the polyglutamine aggregates, is still not universally accepted; the sub-cellular location where aggregate toxicity is expressed is also open to debate; and the mechanism by which aggregates induce neuronal death is still a mystery. Technical factors limit the ability of many studies to conclusively test the role of polyglutamine aggregates in triggering disease pathology. Cell and animal models require over-expression of soluble, monomeric polyglutamine forms as necessary precursors to aggregate formation, making it difficult to draw conclusions about the relative toxicities of native monomer, misfolded monomer, aggregation intermediates, or some form of mature aggregate. It is also difficult to detect and quantify the small

aggregates that may be the most potent toxic forms of polyglutamine (Chen, Berthelier et al. 2001).

In this laboratory, biochemical studies directly focused on the polyglutamine peptides have uncovered some important and fundamental features of polyglutamine peptides: first, a comprehensive protocol of solubilizing and disaggregating polyglutamine peptides make it possible to conduct aggregation studies *in vitro* (Chen and Wetzel 2001); second, studies on polyglutamine aggregation behavior *in vitro,* including the development of a microtiter plate assay for polyglutamine aggregate elongation (Berthelier, Hamilton et al. 2001), suggest a recruitment mechanism of cytotoxicity (Chen, Berthelier et al. 2001); third, detailed studies characterizing the amyloid-like features of polyglutamine aggregates and their assembly kinetics (Chen, Berthelier et al. 2002), including indications that the age-of-onset of these diseases is linked to polyglutamine aggregation nucleation (Chen, Ferrone et al. 2002). These discoveries put us in a position of developing a new cell model system and to thereby study the cellular mechanisms of polyglutamine diseases from a different direction, which could overcome the technical barriers of the existed model systems. For the first time, we directly introduced polyglutamine aggregates prepared *in vitro* from simple polyglutamine peptides into the cytoplasm or nucleoplasm of cells in culture to study the effects on the cells.

Chapter 3 describes our efforts to overcome the challenge of getting polyglutamine aggregates into mammalian cells. Chapter 4 describes the results of comprehensive cytotoxicity studies, in which aggregates are directed to either the cytoplasm or

nucleoplasm. Chapter 5 describes the extension of this cytotoxicity study to include the effects of soluble polyglutamine peptides in this system. Chapter 6 describes further studies to better understand the cell death mechanism observed in our model system. Chapter 7 describes the use of our cell model to evaluate the ability of a recently discovered (A. Thakur and R. Wetzel, unpublished) polyglutamine elongation inhibitor to inhibit polyglutamine cytotoxicity. Chapter 8 consists of a general conclusion, and chapter 9 contains a detailed material and experimental methods section.

Part of the work described in Chapter 3, 4 and 5 has been published in a paper entitled "Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells", with co-authors John R. Dunlap, Richard B. Andrews and Ronald Wetzel, in *Human Molecular Genetics* 11(23): 2905-17, 2002.

CHAPTER 3

OVERCOMING THE CHALLENGE OF GETTING AGGREGATES INTO CELLS

The properties of *in vitro*-grown aggregates

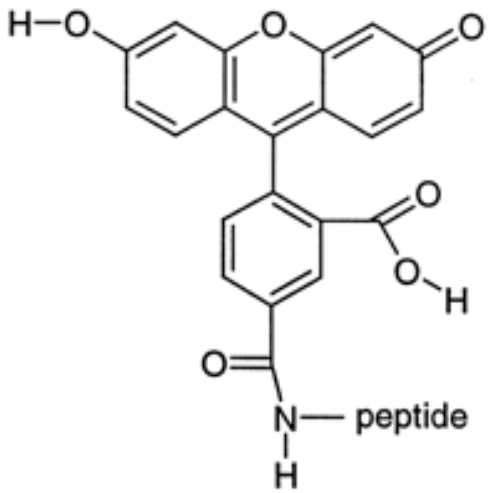
The properties of the peptides used in the experiments

All of the aggregates used in this work were made from controlled *in vitro* aggregation of chemically synthesized peptides. Chen and Wetzel (Chen and Wetzel 2001) demonstrated a method to solublize chemically synthesized polyglutamine peptides, including those in the pathological length range, by using a combination of the organic solvents TFA and HFIP (described in chapter 9). The aggregation of these monomeric peptides was carried out in the test tubes under controlled conditions to generate polyglutamine aggregates that resemble the features of polyglutamine aggregates produced *in vivo* (Chen and Wetzel 2001). Table 8 lists the sequences of all of the peptides used in this study. Peptides were routinely labeled at the N-terminus with fluorescein to provide a means to track the aggregates by microscopy and flow cytometry. Some peptides were also synthesized to include an N-terminal nuclear localization signal (NLS) (Kalderon and Smith 1984) or nuclear export sequence (NES) (Mowen and David 2000). We worked with two types of polyglutamine sequences, one (Q_{20}) shorter than the

Table 8. Synthetic peptides used in the experiments

Peptide Name	Peptide Sequence	MW, Daltons
F-Q_{42}	N^{α}-Fluor-$KKQ_{42}KK_{COOH}$	6,656
F-NLS-Q_{42}	N^{α}-Fluor-PKKKRKVGG-$Q_{42}KK_{COOH}$	7,378
F-NES-Q_{42}	N^{α}-Fluor-LPPLERLTLDGG-$Q_{42}KK_{COOH}$	7,662
F-Q_{20}	N^{α}-Fluor-$KKQ_{20}KK_{COOH}$	3,452
F-NLS-Q_{20}	N^{α}-Fluor-PKKKRKVGG-$Q_{20}KK_{COOH}$	4,175
F-NLS-CspB-1	N^{α}-Fluor-PKKKRKVGG-MLEGKVKWFNSEKGFGFIEVEGKK_{COOH}	4,127
$PGQ_9(P^{2,3})$	N^{α}-$KKQ_9PGQ_4PQ_4PGQ_4PQ_4PGQ_9KK_{COOH}$	5,544

[a]In the Table, "F" and "Fluor" is a fluorescein group, as shown below.

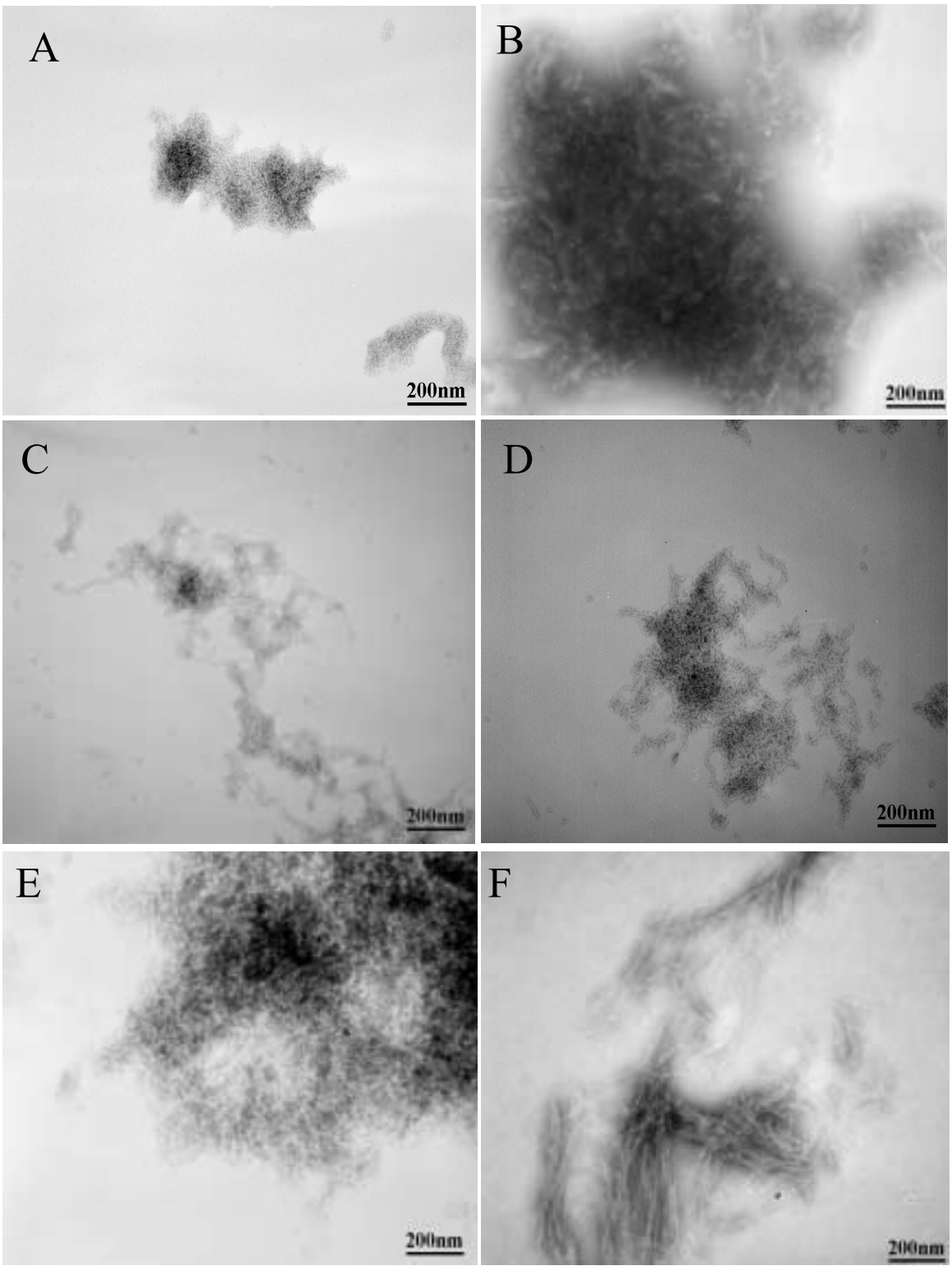


pathological cutoff of 36 glutamines, and one (Q_{42}) longer. Both peptides include flanking pairs of lysine residues to improve the initial solubility of these peptides (Altschuler, Hud et al. 1997) and allow slower, more ordered, *in vitro* aggregation (Chen and Wetzel 2001). For a control peptide, we chose the cold shock protein fragment Csp-B1, which has been shown to form amyloid-like aggregates in solution (Gross, Wilkins et al. 1999). The peptide $PGQ_9(P^{2,3})$ listed in Table 8 is an elongation inhibitor discovered in our laboratory (A. Thakur and R. Wetzel, unpublished) which will be discussed in detail in chapter 7.

The features of the aggregates made in vitro

Studies (Chen, Berthelier et al. 2001; Chen, Berthelier et al. 2002) have shown that polyglutamine aggregates generated in our laboratory possess amyloid-like features, including (1) a high content of β-sheet measured by circular dichroism (CD) spectroscopy; (2) binding to the dye thioflavin-T and altering of its fluorescence properties; (3) binding of Congo red; (4) fibrilar ultrastructures observed by electron microscopy (EM). Csp-B1 aggregates have also been shown previously to exhibit amyloid-like properties (Gross, Wilkins et al. 1999). Figure 7 shows EMs of the aggregates used in this study**.** All the peptides form ordered aggregates of approximately the same width. However, the polyglutamine aggregates are short and curvilinear, while the CspB-1 aggregates are relatively long and straight.

Figure 7. Ultrastructure of *in vitro* aggregates. Aggregates were fixed to mica grids and negatively stained with 0.25% w/v potassium phosphotungstate solution. Micrographs were collected on a Hitachi H600 scanning electron microscope. Shown are aggregates of (A) F-NLS-Q42; (B) F -Q42 (sonicated); (C) F-NLS-Q20; (D) F-Q20; (E) F-NES-Q42; (F) F-NLS-CspB-1.


A
200nm
B
200nm
C
200nm
D
200nm
E
200nm
F
200nm

The stability of the aggregates

These aggregates are stable *in vitro*. Using HPLC to monitor the dissociation of the monomeric peptides (which has the capacity of detecting 2% dissociation), we found that neither Q_{20} nor Q_{42} aggregates dissociate measurably after up to five days incubation at 37 °C in PBS (Figure 8). After five days, the Q_{20} aggregates begin to dissociate slightly, while Q_{42} aggregates show no measurable dissociation; this is consistent with their relative critical concentrations in aggregation (Chen, Berthelier et al. 2001). Csp-B1 aggregates dissociated slightly (5%) after incubation for 3 days under these conditions.

These polyglutamine peptide aggregates also resemble *in vivo* disease protein aggregates in their stability to SDS treatment. Figure 9 shows a Coomassie Brilliant Blue-stained gel with individual lanes loaded with either aggregates or monomers of the Q_{20} and Q_{42} peptides, after 3 minutes in boiling SDS gel loading buffer. The gel shows that no detectable peptide is released from a Q_{42} aggregate and only a small amount ($<10\%$) of peptide is released from a Q_{20} aggregate, suggesting that these aggregates are highly resistant to dissolution in boiling aqueous SDS. In the experiment, all of the samples were loaded at the same amount (5ug), however, the aggregates that could not enter into gel remained in the loading well, and were washed out when opening the gel cassette, with very little still visible at the top of the stacking gel. Although the peptide migration rate in the gel is not as expected for peptides of their molecular weights, as indicated by the standard molecular weight markers, this is probably due to an anomaly in the electrophoretic properties of polyglutamine sequences.

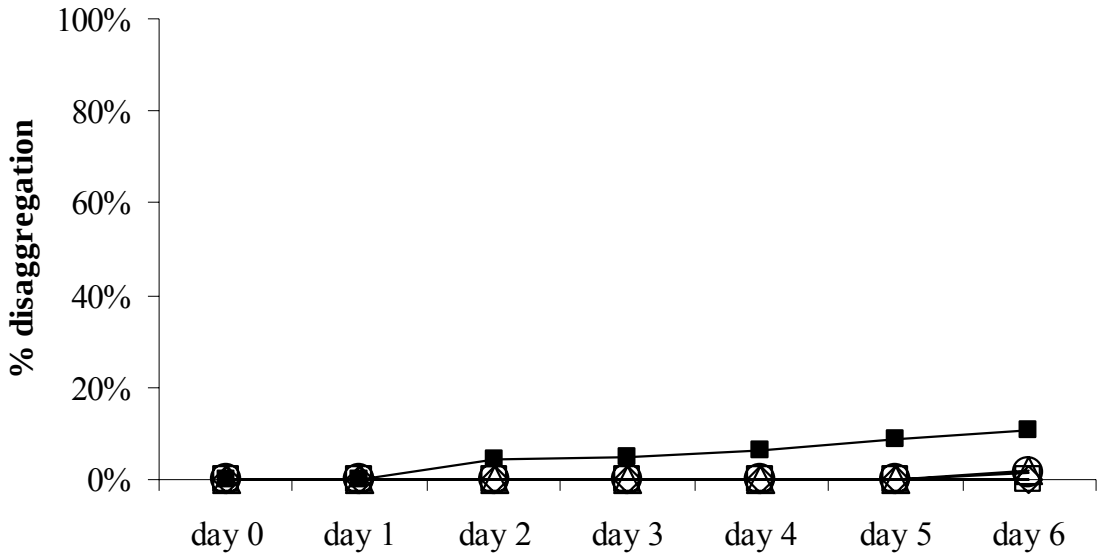


Figure 8. *In vitro* stability of aggregates by monomer dissociation detection in the solution. Different preparations of aggregates were incubated in PBS at a concentration of 10 uM (monomer equivalents) at 37 °C and aliquots were processed as described in Materials and Methods to measure aggregate dissociation. The ordinate shows the percentage of total aggregate-associated peptide released into the supernatant at various incubation times. Open squares, F-NLS-Q42; open diamonds, F -Q42; open circles, F-NLS-Q20; open triangles, F-Q20; filled squares, F-NLS-CspB-1.

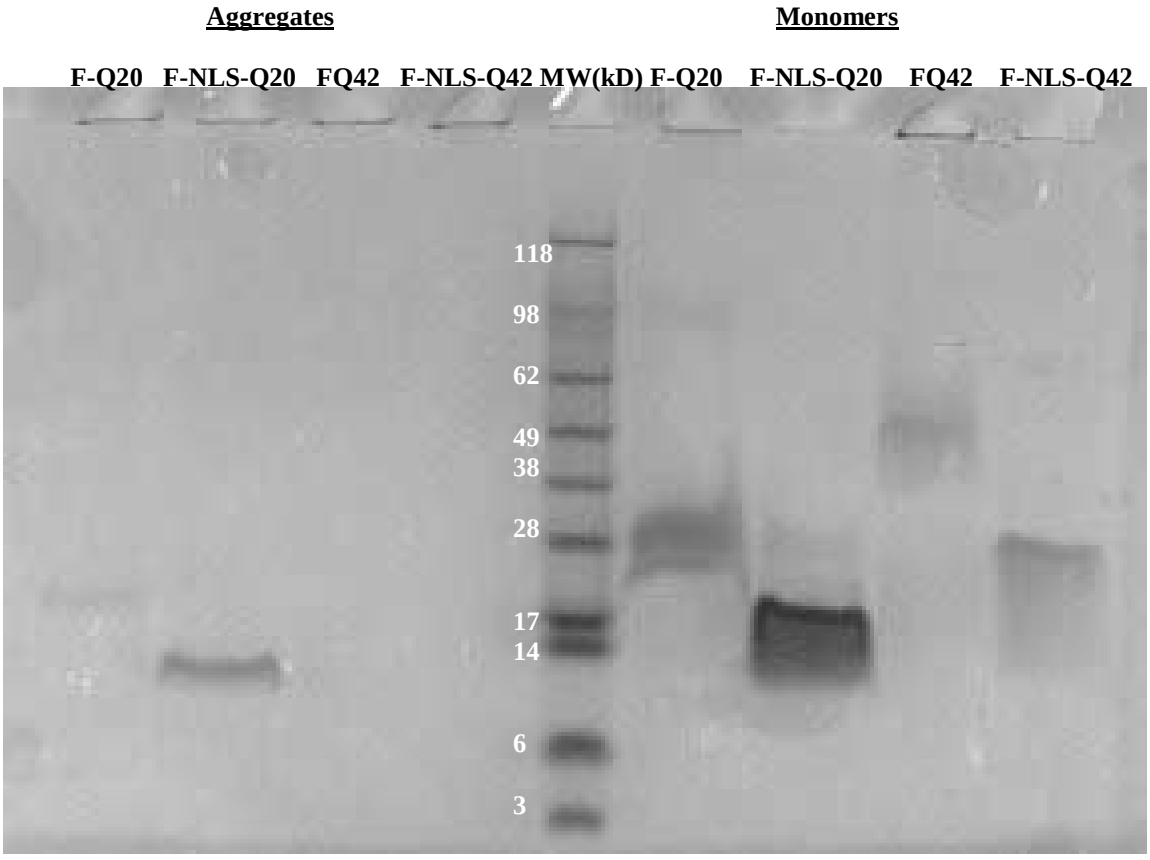


Figure 9. SDS gel for the polyglutamine aggregates and monomers. *In vitro* prepared polyglutamine aggregates (left) and monomers (right) were boiled in 2% SDS loading buffer for 3 min. and then subject to electrophoresis. Monomers were used as control to indicate the dissociation of the aggregates.

Introduction of aggregates into mammalian cells

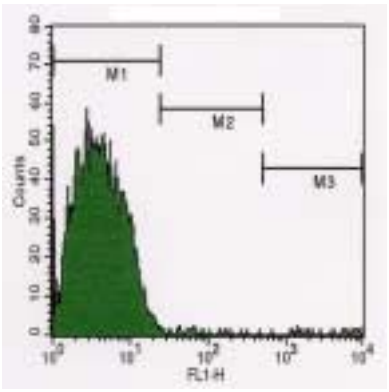
Mammalian cells used in the study

We attempted to introduce these aggregates into mammalian cells. We chose to use both PC-12 cells and Cos-7 cells in our experiments. The PC-12 cell line was established from a rat adrendal gland pheochromocytoma (Greene and Tischler 1976). Induced by nerve growth factor (NGF), PC-12 cells can differentiate into a neuronal phenotype with extended long, branching neuronal-like processes. PC-12 cells, with or without NGF induction, can synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine, and hence have been widely used as a model system for neurobiological studies including Huntington's disease research (Li, Cheng et al. 1999). The Cos-7 cell line is a transformed monkey kidney fibroblast cell line (Gluzman 1981). The idea of including both PC-12 and Cos-7 cells in the experiments is to see whether or not there is a difference between neuronal and non-neuronal cells in response to the aggregate treatment.

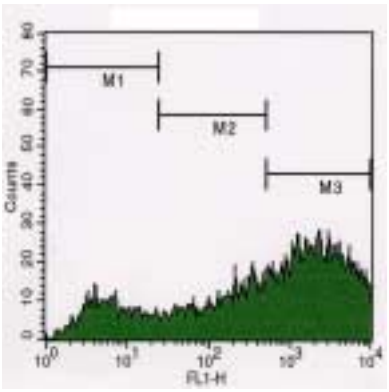
Delivering the aggregates into cells by a liposome-mediated method

We first used a liposome-mediated method to introduce aggregates into cells. The idea is that liposomes can encapsulate small polyglutamine aggregates in PBS solution upon hydration, and then fuse with the hydrophobic cell membrane, delivering the aggregates into the cytoplasm. Flow cytometry studies (Figure 10) showed that it is possible to efficiently deliver aggregates of fluoroscein-tagged Q_{42} polyglutamine

PC-12 cells

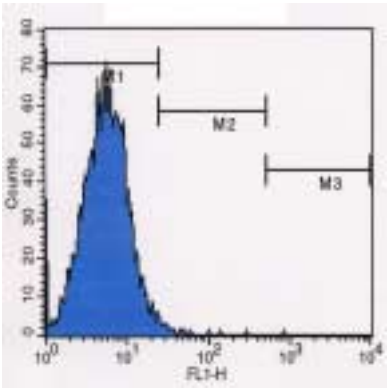


(-) control
M2+M3=0.83%

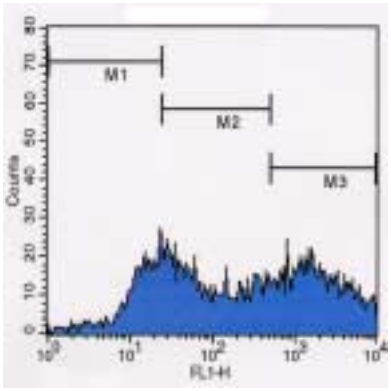


Liposome 4hrs.
M2+M3=84.5%

Cos-7 cells



(-) control
M2+M3=0.48%



Liposome, 4hrs.
M2+M3=79.51%

Figure 10. Introduction of aggregates into cells by the liposome mediated method. PC-12 cells (top series) or Cos-7 cells (bottom series) were treated with (right) or without (left) liposomes encapsulated F-Q_{42} aggregates for 4 hours, which were then washed out and substituted with fresh medium for another 20 hours (right). The cells were collected and analyzed by flow cytometry. The M1 region of each recording represents background fluorescence, the M2 and M3 regions are from fluoroscein fluorescence.

aggregates into both PC-12 and Cos-7 cells by this liposome-mediated method. With both cell types, a four-hour incubation with packaged polyglutamine aggregates results in 80% or more of the cells exhibiting fluoroscein fluorescence.

Aggregates also get into cells by direct incubation method

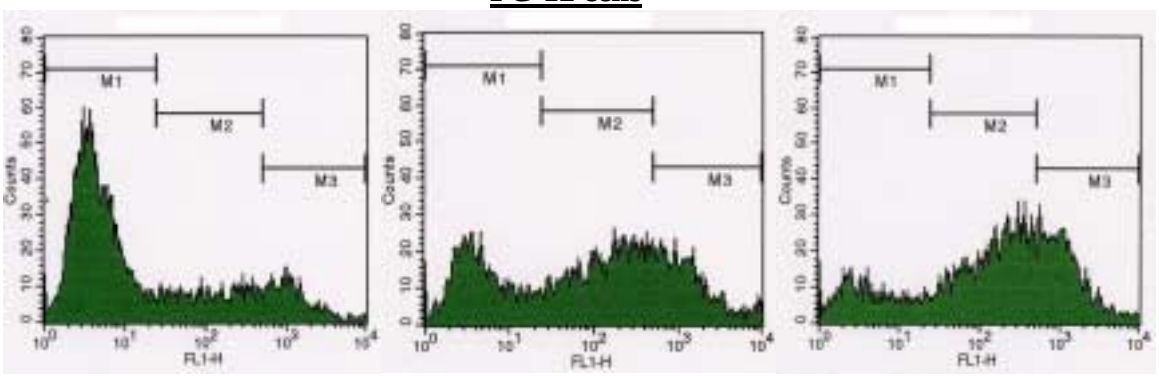
We also included a control experiment for the liposome-mediated method by directly incubating aggregates with cells without liposome packaging. Surprisingly, this control also led to a high percentage of fluorescent cells. Figure 11 shows that although aggregates mixed directly with cells are taken up more slowly, after 24-48 hours 80% or more of cells mixed with aggregates become fluorescent. We decided to conduct further experiments without liposome packaging, in order to reduce the possibility of artifacts associated with added lipids in the cell toxicity studies.

Localization of the aggregates in the cells

Confocal microscopy confirms the aggregates are inside the cells

In order to confirm that aggregates were internalized within cells and not simply adhered to the outer cell surface, we used fluorescence confocal microscopy. It scans individual focal planes of a cell, which very accurately tells whether the aggregates are inside a cell or not. Figure 12 shows micrographs of interior focal planes of both PC-12 and Cos-7 cells that had been incubated with F-Q_{42} aggregates for 24 hours. Figure 12

PC-12 cells

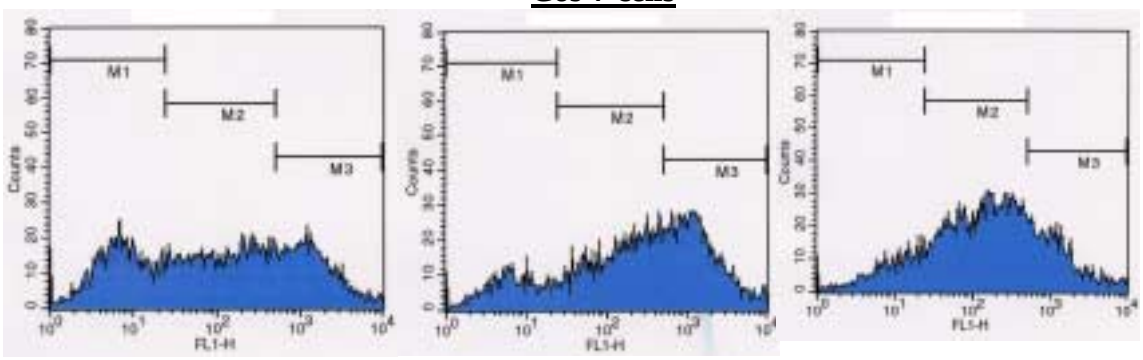


4hrs.
M2+M3=30.08%

24hrs.
M2+M3=67.19%

48hrs.
M2+M3=81.54%

Cos-7 cells



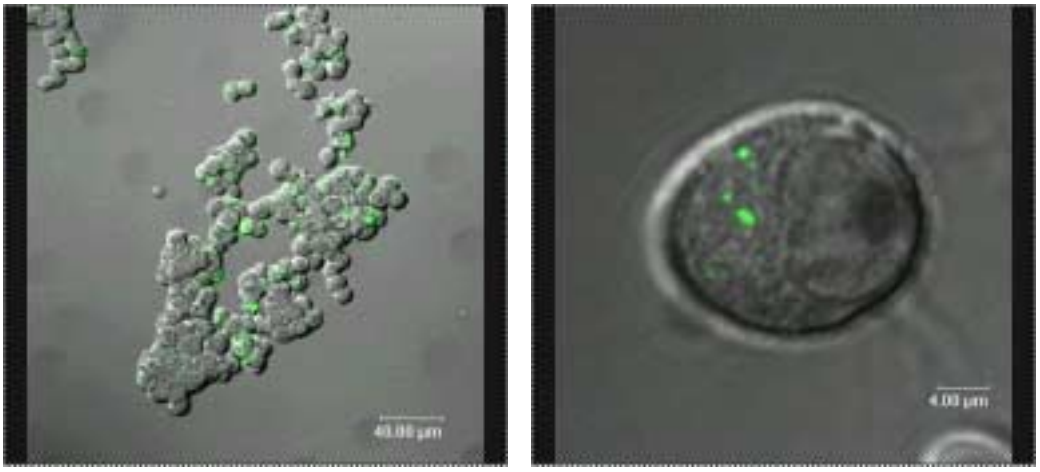
4hrs.
M2+M3=69.81%

24hrs.
M2+M3=84.67%

48hrs.
M2+M3=86.37%

Figure 11. Aggregates getting into cells by direct incubation method. Sonicated F-Q42 aggregates were mixed with PC-12 cells (top series) or Cos-7 cells (bottom series) at various times analyzed by flow cytometry. The M1 region of each recording represents background fluorescence, the M2 and M3 regions are from fluoroscein fluorescence.

PC-12 cells



Cos-7 cells

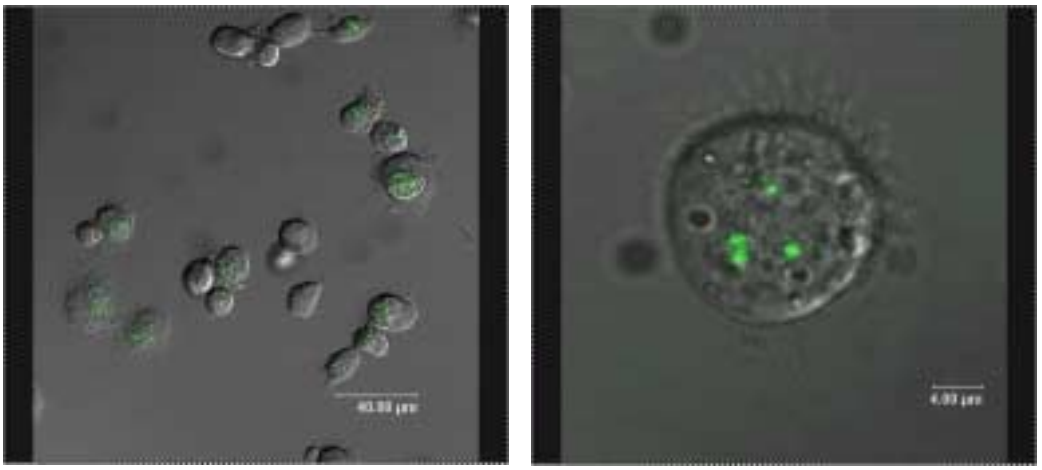


Figure 12. Confocal microscopy of aggregate-treated cells. PC-12 cells (upper) and Cos-7 cells (lower) were treated 24 hours with F-Q_{42} aggregates. Left pictures shows that the aggregates were associated with the cells at a lower magnification. Right pictures clearly show that the aggregates are inside the cells at higher magnification.

clearly shows that these aggregates are located within cells, rather than adherent to the outside of the cells.

Nuclear localization of the aggregates mediated by NLS

In order to probe the dependence of aggregate toxicity on sub-cellular localization, we attempted to deliver them into the nuclei of cells. To do this, we added to some synthetic peptides a sequence consisting of the NLS sequence PKKKRKV (derived from SV40 (Boulikas 1993)) with a fluorescein-tag on the N-terminus and a Gly-Gly spacer on the C-terminus (Table 8). Flow cytometry shows that aggregates of such peptides are taken up into cells about as efficiently as are aggregates without the NLS, as seen in Figure 14-16 in Chapter 4. When the nuclei of these cells are isolated and analyzed by confocal microscopy, fluorescent foci are clearly observed within the nuclear envelope (Figure 13). In contrast, aggregates of F-Q_{42} lacking an NLS accumulate on the nuclear membrane, but do not penetrate into the nucleus (Figure 13).

Sonication and filtration optimized the efficiency by which aggregates are taken up into cell nuclei

To improve the efficiency of cellular and nuclear uptake, we devised methods to produce a smaller effective particle size through sonication and membrane filtration (Chapter 9, Materials and Methods). Figure 13 shows that aggregates of F-NLS-Q_{42} become more efficient at crossing into the cell nucleus as the average particle size of the aggregates gets smaller. Decreasing the particle size of F-Q_{42} aggregates, in contrast, does not alter their fundamental *inability* to penetrate the nucleus. Based on their

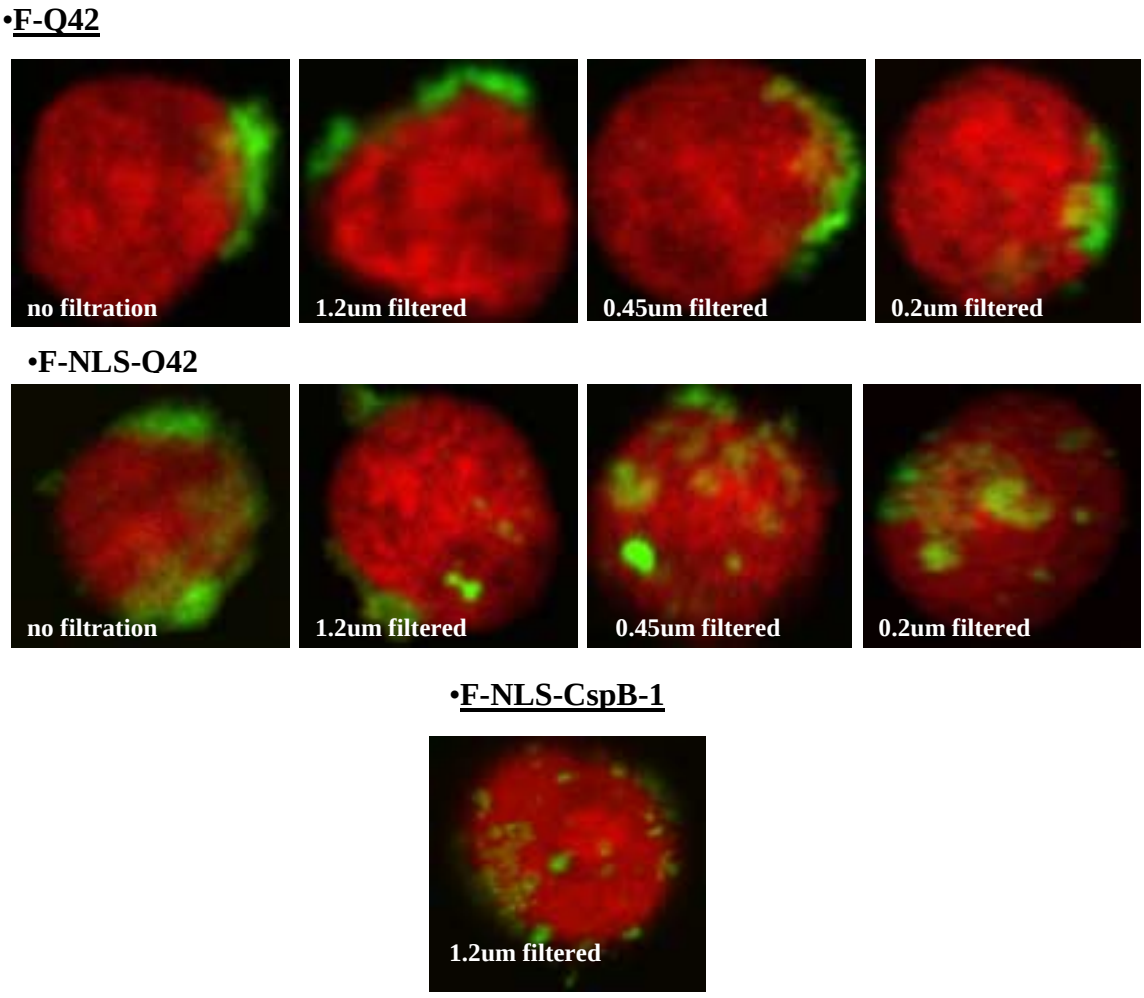


Figure 13. Nuclear localization of the aggregates. Confocal microscopy shows the nuclei extracted from PC12 cells incubated 24 hrs with various aggregates. The upper series show that F-Q_{42} aggregates do not penetrate beyond the nuclear membrane. The middle series show that aggregates of F-NLS-Q_{42} peptides penetrate into the nucleus, with increased efficiency as their size decreases. The lower panel shows that F-NLS-CspB-1 aggregates, sonicated and subjected to a single filtration through a 1.2 um filter, very efficiently penetrate the nucleus.

filtration behavior, aggregates prepared in this way appear to have an average effective size of greater than 100 nm, while the functional diameter of the nuclear pore is normally considered to be no larger than about 26 nm (Talcott and Moore 1999). Nonetheless, the dependence of aggregate uptake into the nucleus on the presence of an NLS strongly suggests that it occurs *via* the NLS-associated nuclear pore. Figure 13 also shows that fluoroscein tagged NLS-CspB-1 aggregates are very efficient at getting into the nucleus. This shows that aggregate transport across the outer and nuclear membranes does not depend on the polyglutamine sequence.

Discussion

Do in vitro aggregates possess the critical features of in vivo aggregates?

Although conditions in the cell are very different from those in a test tube, the aggregates made *in vitro* may in their critical features resemble aggregates produced *in vivo*. First, the aggregates found *in vivo* made up of the mutant protein containing an expanded polyglutamine sequence, and the soluble polyglutamine peptides we generate in our studies, are both chemically and conformationally similar to each other (Chen and Wetzel 2001; Bennett, Huey-Tubman et al. 2002). Second, it has been generally accepted that the process of aggregate formation is mediated through a self-assembly of polyglutamine containing proteins into aggregates (Wanker 2002; Ross, Poirier et al. 2003). Similarly, studies of the aggregation kinetics of the peptides used in our study reveals a nucleation-dependent polymerization that may be the fundamental mechanism of polyglutamine aggregation (Chen, Berthelier et al. 2002; Chen, Ferrone et al. 2002).

Third, the aggregates that we generate *in vitro* present amyloid-like features (Chen, Berthelier et al. 2002), which are also similar to those generated *in vivo* by polyglutamine-containing protein, at least according to some reports (Davies, Turmaine et al. 1997). Fourth, aggregates of ataxin 3 (Chen, Berthelier et al. 2002), as well as aggregates isolated from HD brain tissue (Berthelier, unpublished), have the ability, in analogy to synthetic polyglutamine aggregates to serve as a matrix for polyglutamine elongation. Taken together, the similarity of the aggregates we make *in vitro* to the aggregates generated in cells supports the relevance of our aggregate toxicity.

How are the in vitro produced protein aggregates taken up by cells and cell nuclei?

It is of great interest how the protein aggregates described above, despite their large sizes, are so efficiently taken up by cells and delivered into cell nuclei. This surprising result is not restricted to polyglutamine sequences, since we observed similar or greater nuclear uptake of Csp-B1 aggregates. All of the aggregates described here exhibit amyloid-like structures in the EM and carry a net positive charge at neutral pH; it is possible that one or both of these features may contribute to their efficient membrane transport. Since multiple localization signals are thought to facilitate passage of large substrates through the pore (Talcott and Moore 1999), the fact that fibrils made of NLS-tagged peptides consequently contain significant numbers of NLS, explain the ability of these aggregates to gain passage through the nuclear pore. Our results do suggest that even relatively large aggregates of polyglutamine and other sequences can be transported across the nuclear membrane in a process mediated by an NLS. This suggests that it is

possible that in the disease mechanism polyglutamine aggregates containing crytic NLS sequences [or co-aggregates between disease proteins and other polyglutamine proteins that contain NLS sequences (Preisinger, Jordan et al. 1999)] might form in the cytoplasm and then be transported into the nucleus.

It is formally possible that aggregate dissociation to monomers, followed by reaggregation, might contribute to cell or nuclear uptake of the aggregates. However, the results from *in vitro* aggregate stability studies suggested that the aggregates are very stable for the times and conditions of the treatment (Figure 8). It is very unlikely that the observed ability of these aggregates to be transported across cell and nuclear membranes is due to a disassembly-reassembly pathway; this is especially true for the F-NLS-Q_{20} aggregates, since Q_{20} peptides would require high concentrations and long incubation times to re-aggregate (Chen, Berthelier et al. 2001). If Q_{20} aggregates had disassociated during incubation with the cells, it would be impossible for them to re-aggregate under these conditions.

The problems and limitations in getting the aggregates into cells

Although aggregates can be delivered into cells by the direct incubation method, there are some problems associated with it. First, there's no control over how much aggregate gets into cells and nuclei. So it is possible that some cells take up more aggregates than others, leading to different responses. Thus, in response to the same treatment, one may see a wide range of responses among the cells, from cells with severe effects (due to high concentration of aggregates inside the cells or nuclei) to cells with little or no effects (because there are little or no aggregates inside the cells or nuclei).

Second, there is no way to synchronize the uptake of aggregates by all the cells. We know that it takes about 24-48 hours for 80% of cells to acquire the aggregates. But there may be cells that take up aggregates in only 4 hours and quickly exhibit effects, while at this time other cells may be without aggregates and without effects. Thus the time course of the effects we observed reflects the system as a whole. Third, we have observed that in the experiments, there are a large amount of big aggregates attached to the membrane of the cells, presumably because the aggregates, which carry a net positive charge, are attracted to the negatively charged cell membrane. Sonication and filtration to decrease the size of the aggregates seems to help to reduce this effect. But, despite this, the aggregates tend to re-associate together into large clusters again, after filtration, during incubation with the cells. This effect is especially strong in higher concentration treatments. These large aggregates are not delivered into the cells, but give false signals for cells containing aggregates in the flow cytometry determination and also made it impossible to measure how much aggregate is in the cells by flow cytometry or by fluorescence detection methods.

One way to improve these experiments would be to control delivering of aggregates to the cells. One could, for example, use microinjection to directly inject aggregates into cells in the future. Another improvement would be to devise a way to measure aggregate uptake directly on a cell-by-cell basis. For example, one might use cells transfected with construct for low level of expression of a huntingtin (htt) [with moderate length of polyglutamine (~30) that does not aggregate rapidly] fusion to a fluorescent protein (other than green) for treatment with fluorescein-tagged polyglutamine aggregates. If the

expressed peptide is recruited to the aggregates, it would cause FRET (fluorescence resonance energy transfer), which can be measured. The amount of FRET experienced by the expressed protein could be related to the amount of aggregates taken up; this could be done on a cell-by-cell basis using flow cytometry.

CHAPTER 4

TOXICITY OF AGGREGATES INTRODUCED INTO THE CYTOPLASM AND NUCLEOPLASM

Initial studies on cytotoxicity of the aggregates using flow cytometry

Toxicity of cytoplasmic aggregates

From the confocal microscopy study, we observed that the majority of the aggregates of the F-Q_{45} peptide lacking an NLS are located in the cytoplasm. We then further studied the effect of these aggregates on cell viability. We used propidium iodide (PI) exclusion, a standard staining method for distinguishing viable from non-viable cells. If PI is able to penetrate to the nucleus due to damage to cell membranes, it intercalates into nucleic acid and by doing so (a) concentrates in the nucleus and (b) exhibits enhanced fluorescence (Slezak and Horan 1989) detectable in flow cytometry and confocal microscopy. Figure 14 shows that PC-12 cells efficiently take up F-Q_{42} aggregates but remain viable, despite their aggregate burden, with only 12% of the cells (essentially identical to the control 11%) exhibiting PI staining. The results are the same for Cos-7 cells, with 12% of cells dying in the presence of cytoplasmic aggregates, essentially identical to the control value of 10% cell death without aggregates. This data

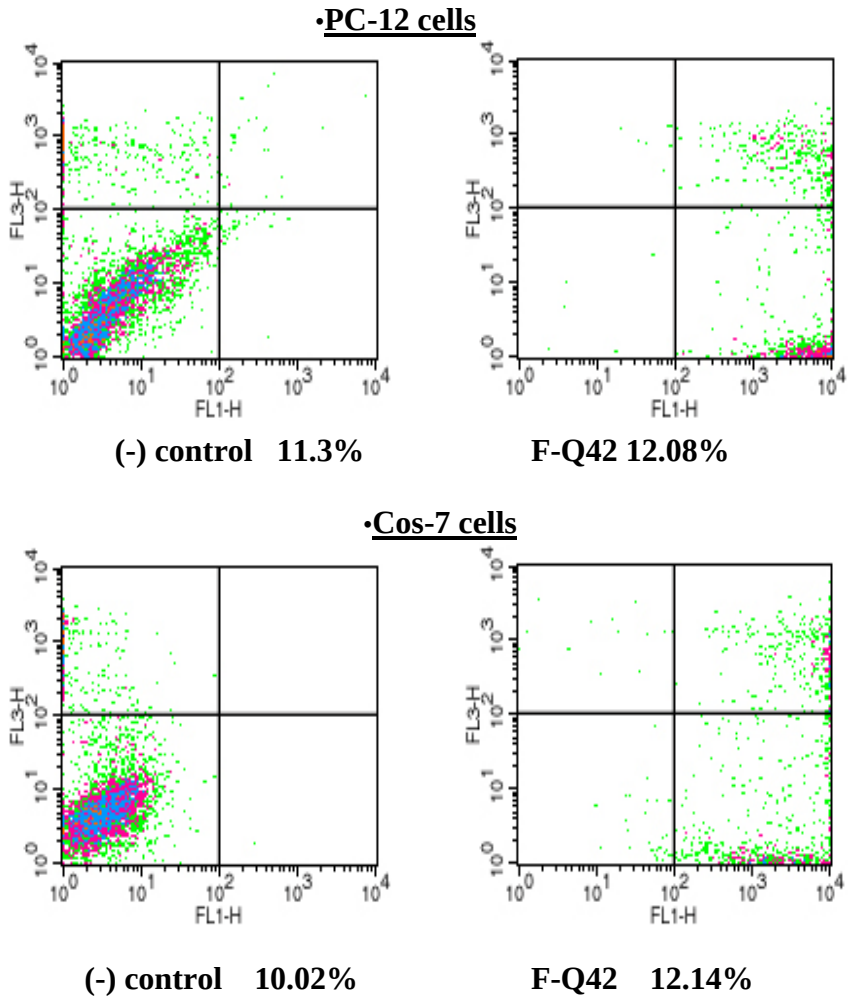


Figure 14. Flow cytometry on cell viability of cytoplasmic aggregates. PC-12 cells (top series) or Cos-7 cells (bottom series) were incubated with (right) or without (left) F-Q_{42} aggregates for 24 hrs, then stained with propidium iodide and analyzed by flow cytometry. The individual panels show the result of one experiment each in which 1.2 um filtered aggregates were mixed with cells and evaluated for both cell uptake of aggregates (FL-1) and cell death (FL-3). Panel labels reflect percent cell death by PI staining.

suggests that polyglutamine aggregates are not toxic to the cells when they are in the cytoplasm.

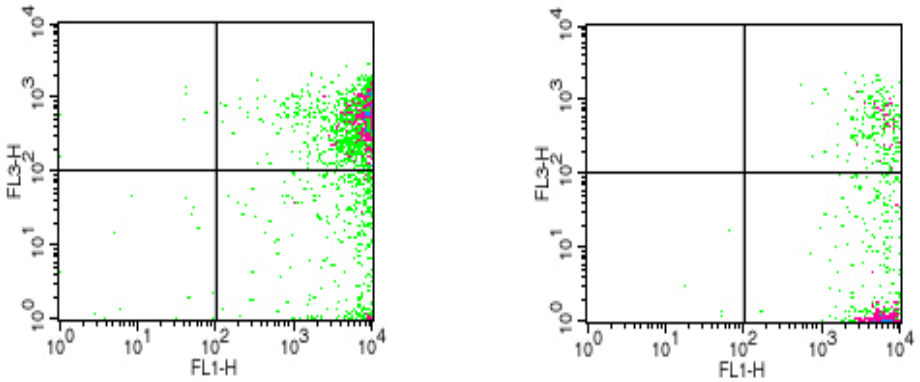
Toxicity of intranuclear aggregates

In contrast, when cells were treated with F-NLS-Q_{42} aggregates capable of penetrating into nuclei (as observed in confocal microscopy), 65% of the PC-12 cells and 66% of Cos-7 cells exhibit PI fluorescence and are hence non-viable (Figure 15). We then wanted to test whether or not this nuclear toxic effect is restricted to polyglutamine aggregates. As a control peptide for the cytotoxicity experiments, we added an NLS sequence to Csp-B1, a peptide fragment of a bacterial protein previously shown to make amyloid fibrils *in vitro* (Gross, Wilkins et al. 1999). This modified peptide remains capable of forming amyloid fibrils (Figure2) and the aggregates are as efficient as polyglutamine aggregates in being taken up by cells and delivered into cell nuclei (Figure 13). Figure 15 shows that, in spite of this efficient nuclear uptake, F-NLS-Csp-B1 aggregates are not toxic to Cos-7 or PC-12 cells. Thus, the toxicity of intranuclear aggregates in these experiments seems to require the presence of the polyglutamine sequence.

Toxicity of aggregates of a polyglutamine sequence under the pathological threshold: Q_{20}

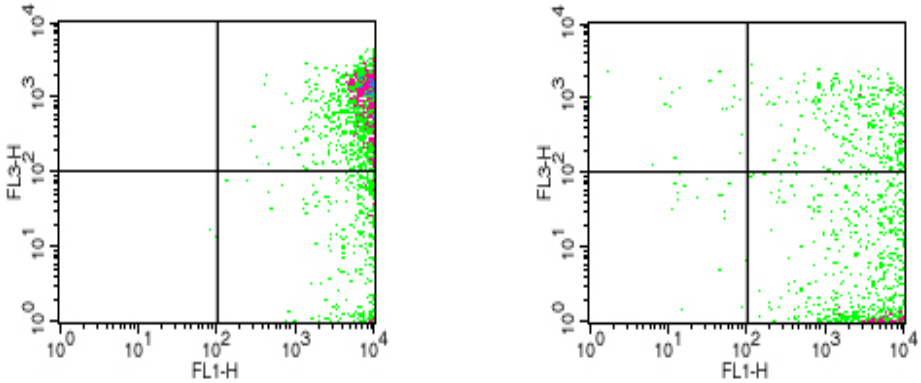
We then addressed the question of why there is a repeat length cutoff in the disease onset. Is this due to the difference in the toxicity of the polyglutamine aggregates made from different glutamine lengths, or in the tendency of different

•PC-12 cells



F-NLS-Q42 65.14% **F-NLS-CspB-1 16.67%**

•Cos-7 cells



F-NLS-Q42 66.36% **F-NLS-CspB-1 16.66%**

Figure 15. Flow cytometry on cell viability of nuclear aggregates. PC-12 cells (top series) or Cos-7 cells (bottom series) were incubated with F-NLS-Q42 aggregates (left) and F-NLS-CspB-1 aggregates (right) for 24 hrs, then stained with propidium iodide and analyzed by flow cytometry. The individual panels show the result of one experiment each in which 1.2 um filtered aggregates were mixed with cells and evaluated for both cell uptake of aggregates (FL-1) and cell death (FL-3). Panel labels reflect percent cell death by PI staining.

polyglutamine lengths to aggregate? Although Q_{20} peptides form aggregates very sluggishly at 37 °C (Chen, Berthelier et al. 2001), it is possible to produce Q_{20} aggregates *in vitro* by exploiting the process of freeze-concentration (Chen, Berthelier et al. 2002). We treated both PC-12 cells and Cos-7 cells with Q_{20} aggregates at the same molar concentration (that is, as expressed by the molar concentration of monomers in the aggregates) with Q_{45} aggregates. Figure 16 shows that aggregates of F-NLS-Q_{20} kill both PC-12 and Cos-7 cells about as effectively as do aggregates of F-NLS-Q_{42} (Figure 9). Figure 16 also shows that aggregates of Q_{20} peptides lacking an NLS exhibit poor cytotoxicity, in analogy to the Q_{42} result. These observations strongly support the view that the pathological length cutoff for polyglutamine disease onset is due to the inefficiency of the aggregation process itself for short polyglutamine peptides. Once aggregates are formed, they are equally toxic regardless of the repeat length of their constituent peptides.

A summary of the cytotoxicity results obtained by flow cytometry

Figure 17 summarizes the results of three to eight (depending on the aggregates used) independent experiments conducted using flow cytometry evaluated PI uptake as a marker for cell death. Statistical analysis of the data confirms that aggregates of both long and short polyglutamine peptides tagged with an NLS are highly cytotoxic, while cells treated with aggregates of identical peptides lacking the NLS exhibit essentially the same low amount of cell death as do untreated control cells. Aggregates of the NLS-tagged CspB-1 peptide are similarly non-toxic.

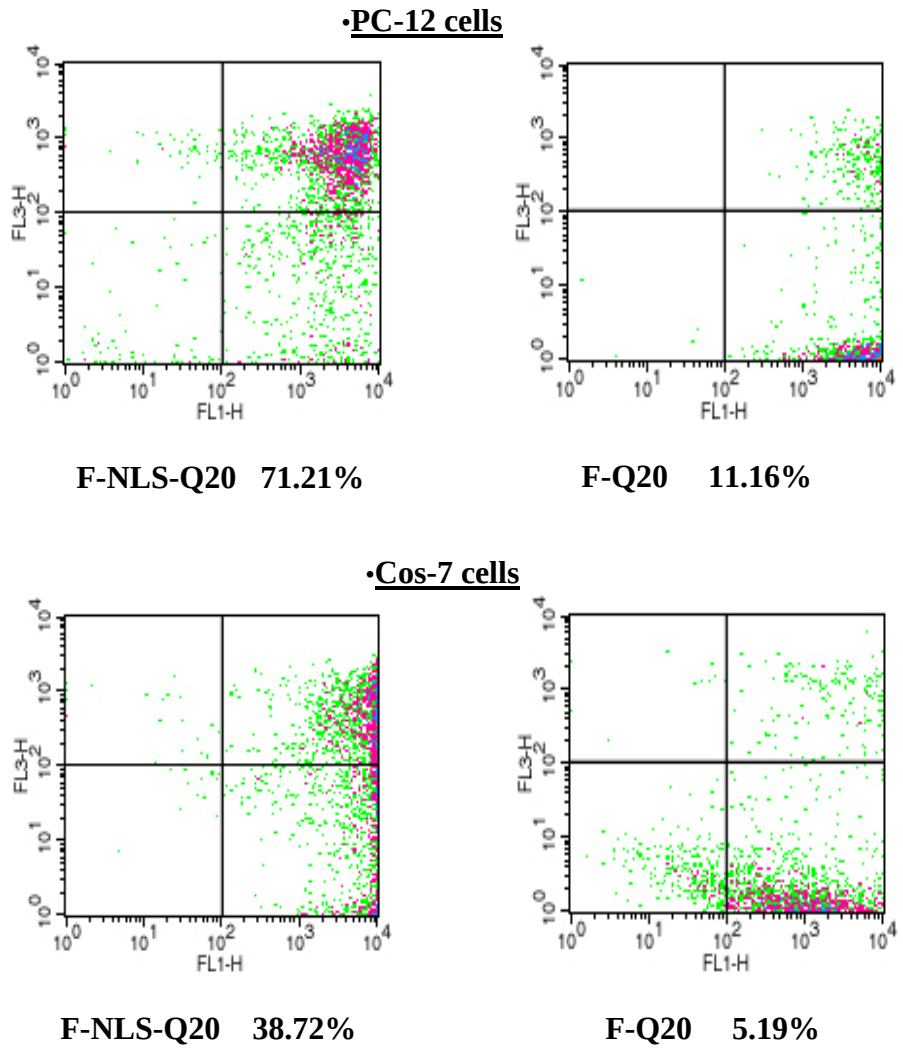


Figure 16. Flow cytometry on cell viability of short (Q20) aggregates. PC-12 cells (top series) or Cos-7 cells (bottom series) were incubated with F-NLS-Q20 aggregates (left) and F-Q20 aggregates (right) for 24 hrs, then stained with propidium iodide and analyzed by flow cytometry. The individual panels show the result of one experiment each in which 1.2 um filtered aggregates were mixed with cells and evaluated for both cell uptake of aggregates (FL-1) and cell death (FL-3). Panel labels reflect percent cell death by PI staining.

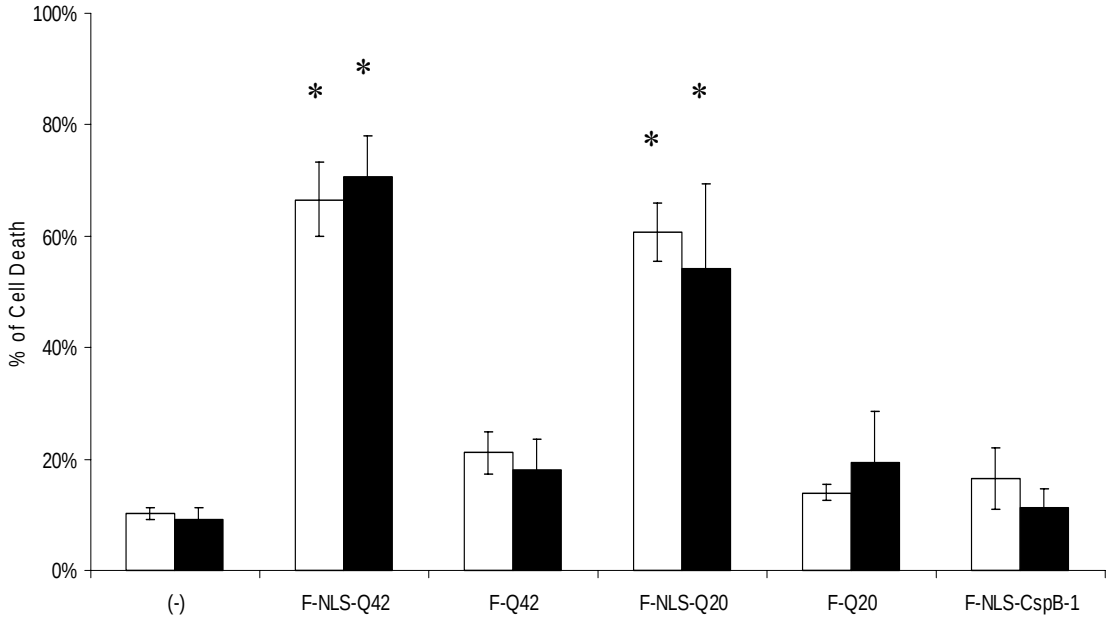


Figure 17. A summary of propidium iodide flow cytometry data on the cytotoxicity of the aggregates. PC-12 (open bars) or Cos-7 (closed bars) cells were treated with various aggregates indicated in the figure. Three to eight independent experiments were summarized here and the asterisk indicates data in which $p \leq 0.001$ compared to control.

Cell viability study by MTS reduction assay

In order to confirm the results obtained in the flow cytometry study and eliminate the bias that might be obtained from relying on only one method, we carried out additional assays to evaluate the viability of cells treated with aggregates. The MTS reduction assay is based on measuring mitochondrial function as an indirect way to assess cell viability (Gschwind 1997). Consistent with the flow cytometry results, Figure 18 shows that the viability of both Cos-7 and PC12 cells treated with NLS-polyglutamine aggregates is significantly reduced compared to cells treated with polyglutamine aggregates without an NLS or with non-polyglutamine aggregates with an NLS (F-NLS-CspB-1) (which are essentially the same as untreated control cells). The difference in the degree of cytotoxicity measured for NLS-polyglutamine aggregates using flow cytometry (over 50% for PC-12 cells and over 60% for Cos-7 cells) versus MTS reduction (about 30% for PC-12 cells and about 40% for Cos-7 cells) can be explained by the difference in the sensitivities of different assays. Qualitatively, the data obtained from the MTS reduction assay confirms the cytotoxicity results observed by flow cytometry/PI exclusion.

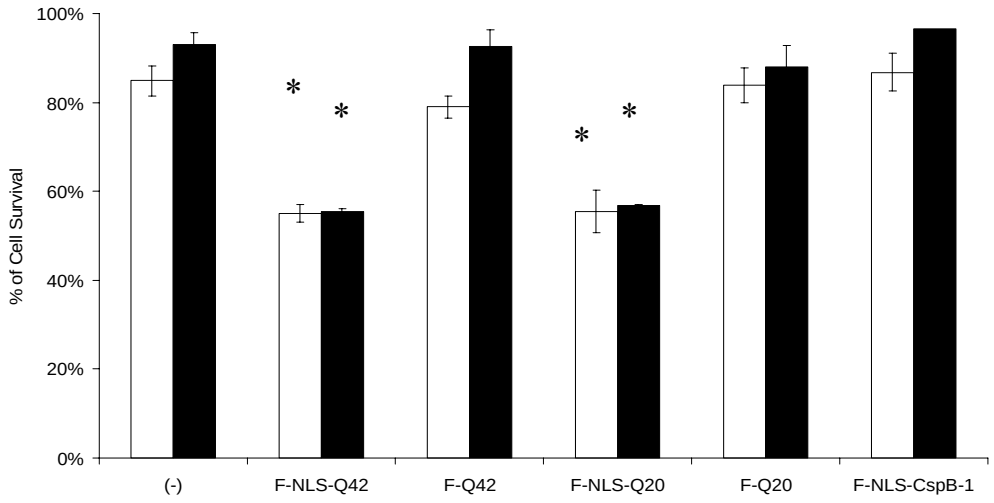


Figure 18. MTS reduction assay on the cell viability after aggregates treatments. PC-12 (open bars) or Cos-7 (closed bars) cells were treated with various aggregates indicated in the figure. Note that the ordinate here is cell survival rather than cell death. Data shows the mean and standard error of two to four experiments, except for the F-NLS-CspB-1/Cos-7 experiment, which is from a single experiment. The asterisk indicates data in which $p \leq 0.001$ compared to control.

Cytotoxicity study by LDH release assay

A summary on cytotoxicity study using the LDH release assay

The mechanism of the LDH release assay is similar to that of the PI exclusion assay. The LDH assay measures the release of a cytoplasmic enzyme, lactate dehydrogenase (LDH), into the medium when cell membrane integrity is lost. The advantage of this assay over PI exclusion is that it can easily follow the course of cytotoxicity quantitatively without disrupting the cells. The percentage of cell death in a cell culture is represented by LDH activity measured in the supernant relative to the total possible LDH, determined after complete cell lysis (Gschwind and Huber 1995). Figure 19 summarizes 2-4 independent experiments using the LDH release assay. The results are consistent with the results obtained by both flow cytometry/PI exclusion and by MTS reduction, with significant cytotoxicity only detected in NLS-polyglutamine treated cells. There is no significant difference in the results for PC-12 and Cos-7 cells. Again, because different assays measure different aspects of cell death, the increased cytotoxicity in NLS-polyglutamine treated cells (about 30% for PC-12 and about 50% for Cos-7) measured by the LDH release assay is slightly different from the other two methods. But the overall conclusion remains the same.

Dose-response study of polyglutamine aggregate cytotoxicity

Having established that all these measures of cell death give equivalent results, we took advantage of the relative ease of the LDH release assay to study the cytotoxicity

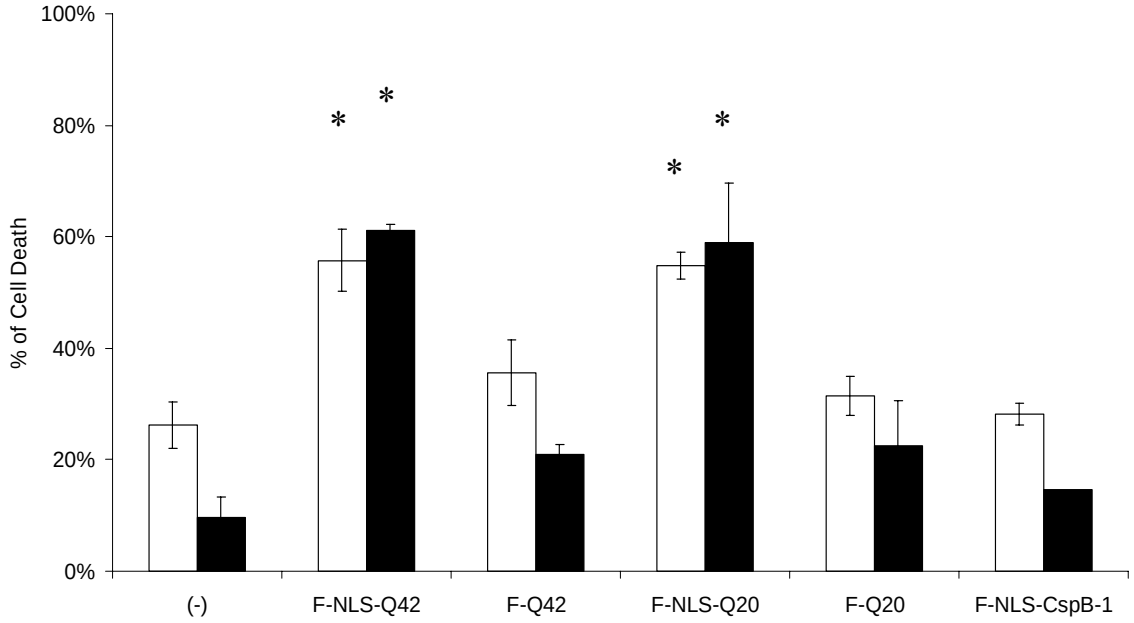


Figure 19. LDH releasing assay on the cytotoxicity of various aggregates. PC-12 (open bars) or Cos-7 (closed bars) cells were treated with various aggregates indicated in the figure. Data shows the mean and standard error of two to four experiments. The asterisk indicates data in which $p \leq 0.001$ compared to control.

of aggregates at different doses. We used only PC-12 cells in these studies, since we observed essentially no difference between the two cell lines in the cytotoxicity assay as described above. Figure 20 shows that aggregates of both F-NLS-Q_{42} and F-NLS-Q_{20} exhibit similar dose-response curves in the LDH-release assay, with mid-points in the low uM range. At the same time, aggregates of polyglutamine peptides lacking the NLS, as well as the nuclear-targeted non-polylutamine control peptide F-NLS-CspB-1, are completely non-toxic up to 100 uM concentration or higher. Aggregates of a Q_{42} peptide containing a nuclear export signal, F-NES-Q_{42} (Table I), were similarly non-toxic up to a 100 uM concentration (not shown). The lack of toxicity of these control peptides, even at very high concentrations, emphasizes the significance of the efficient cell killing observed for the nuclear targeted aggregates. There are several possible explanations for the slightly higher potency of the F-NLS-Q_{42} aggregates compared to F-NLS-Q_{20} aggregates shown in Figure 20. These include the two-fold greater mass, a possibly higher efficiency of cellular and/or nuclear uptake, and a lower tendency to disaggregate (Figure 3), of Q_{42} compared to Q_{20} aggregates.

Time-course studies of polyglutamine aggregate cytotoxicity

Figure 21 shows the time course of cell death, as measured by the LDH release assay, for cells treated with various aggregates. In agreement with other results described here, nuclear-localized aggregates of both Q_{20} and Q_{42} peptides are toxic. The time course of cell death induced by toxic aggregates is consistent with the time course for aggregate internalization. Thus, Figure 11 shows that substantial uptake of polyglutamine aggregates occurs at some time between four and 24 hours. This is consistent with the

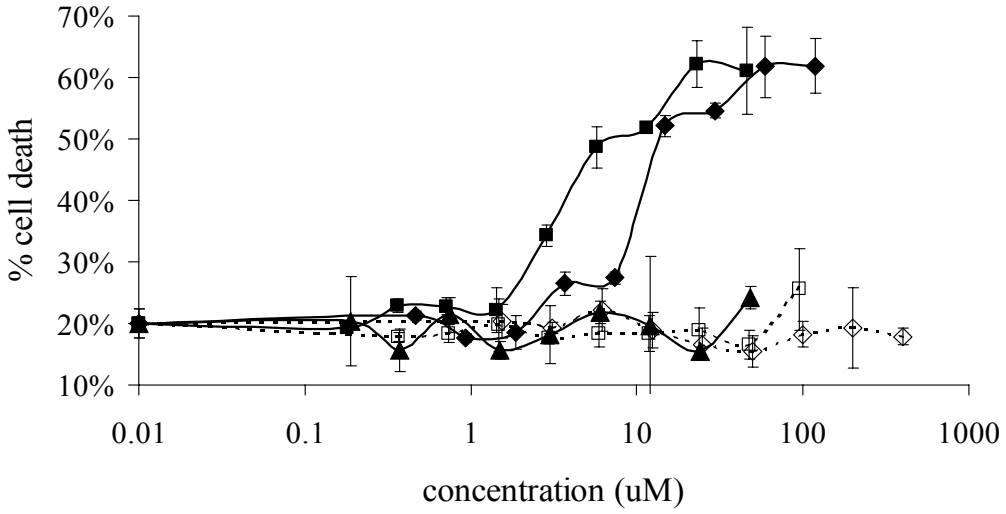


Figure 20. Dose-dependent cytotoxicity of polyglutamine aggregates. PC-12 cells were incubated with various concentrations of poyglutamine aggregates and cell death measured by the LDH-release assay. F-NLS-Q_{42}, closed squares (■); F-Q_{42}, open squares (□); F-NLS-Q_{20}, closed diamonds (◆); F-Q_{20}, open diamond (◇); F-NLS-CspB-1, closed triangles (▲). The data reflects the mean values of 2-4 independent repetitions, each conducted in triplicate; data was normalized to a value of 20% cell death for the untreated cells. The average EC_{50} for the F-NLS-Q_{42} aggregates is about 3-4 uM, and for F-NLS-Q_{20} aggregates 12-15 uM.

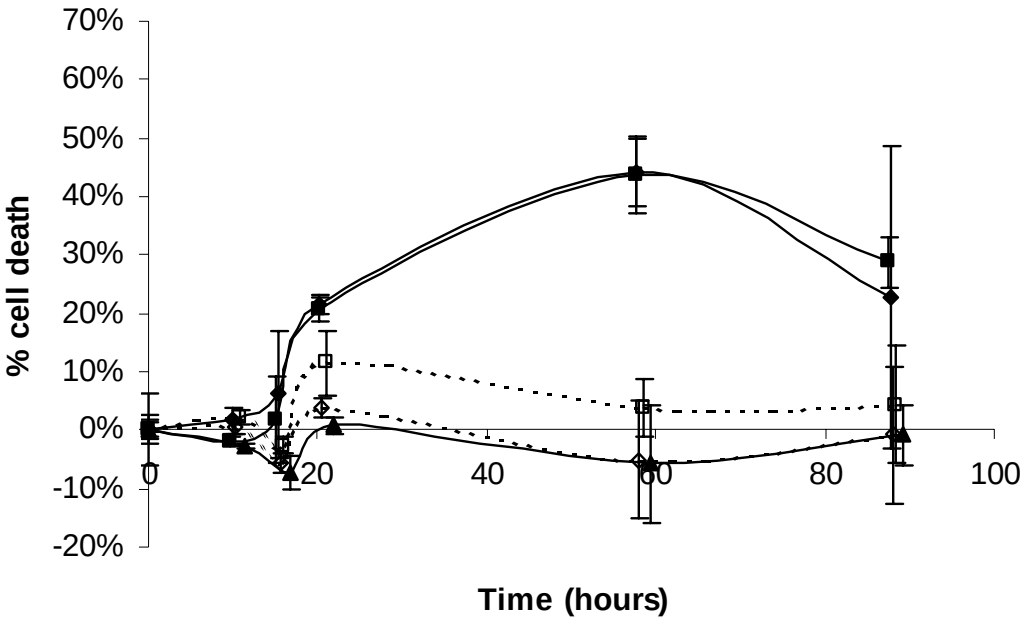


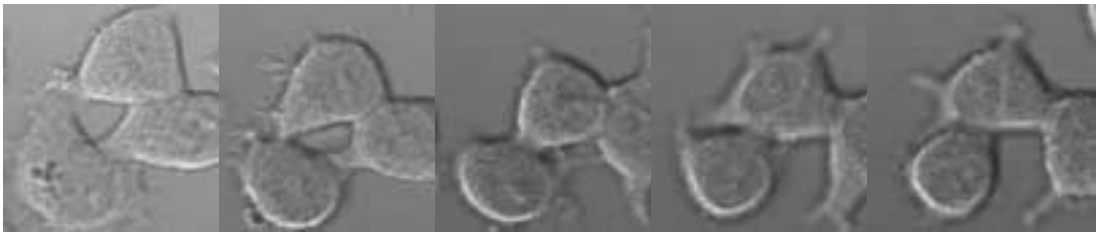
Figure 21. Time course of the cytotoxicity of polyglutamine aggregates and monomers. PC-12 cells were incubated with polyglutamine aggregates for over 90hours and cell death was measured by the LDH-release assay at various time points. F-NLS-Q_{42}, closed squares (■); F-Q_{42}, open squares (□); F-NLS-Q_{20}, closed diamonds (◆); F-Q_{20}, open diamond (◇); F-NLS-CspB-1, closed triangles (▲). Two independent experiments were conducted, each in triplicate; data was normalized by subtracting out the control value at each time point, so that percent cell death means the increased cell death compared to control.

results shown in Figure 21 that show an onset of cell killing between 15 and 18 hours after initiation of the experiment. Polyglutamine aggregates localized in the cytoplasm and non-polyglutamine aggregates localized in the nucleoplasm are not toxic for up to 90 hours, as shown in Figure 21. In the LDH assay, the cell medium was normally maintained in a serum free state to avoid both a high LDH background associated with the serum, and the variation in that background observed for serum purchased at different times (this will be discussed in Chapter 9). This, however, led to more extensive death in control cells, so that the majority of cells are dead after 3 to 4 days due to serum starvation. This limited the time course study to 90 hours. Except for the increased background of cell death caused by serum starvation in all the experiments, the increased cytotoxicity caused by NLS-polyglutamine aggregates is essentially the same as in the previous studies using the flow cytometry/PI exclusion method.

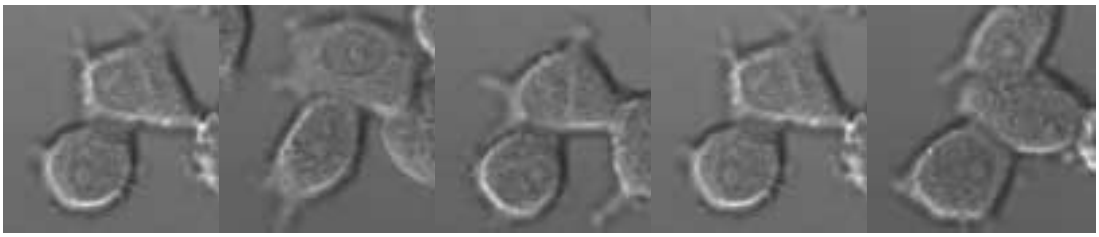
A live cell system monitoring a single cell for aggregate toxicity

We attempted to visualize the dynamic structural changes occurring in cells treated with toxic aggregates. A live cell system was set up on the confocal microscope station to monitor and record cells in the process of taking up aggregates and undergoing cell death. In Figure 22, serial pictures from this system are shown for control cells maintained in the condition of high antibiotics, with mineral oil covering and a bottom heating plate for a course of 24 hours. As shown in Figure 22, cells can be maintained viable under these conditions, making possible subsequent experiments. We then used the system to monitor cells treated with F-NLS-Q_{20} aggregates. Figure 23 shows pictures of selected time points of a single cell during the 24 hour time course. The data, suggests

(-) control



00 hrs. 2.5 hrs. 5 hrs. 7.5 hrs. 10 hrs.



11.67 hrs. 15.83 hrs. 17.5 hrs. 20 hrs. 21.5 hrs.

Figure 22. A live cell microscopic system monitoring control cell morphology over a 24 hrs period. PC-12 cells were maintained in the live cell system for 24 hours and confocal images were acquired at various time points as indicated in the figure.

F-NLS-Q20 Aggregate treated

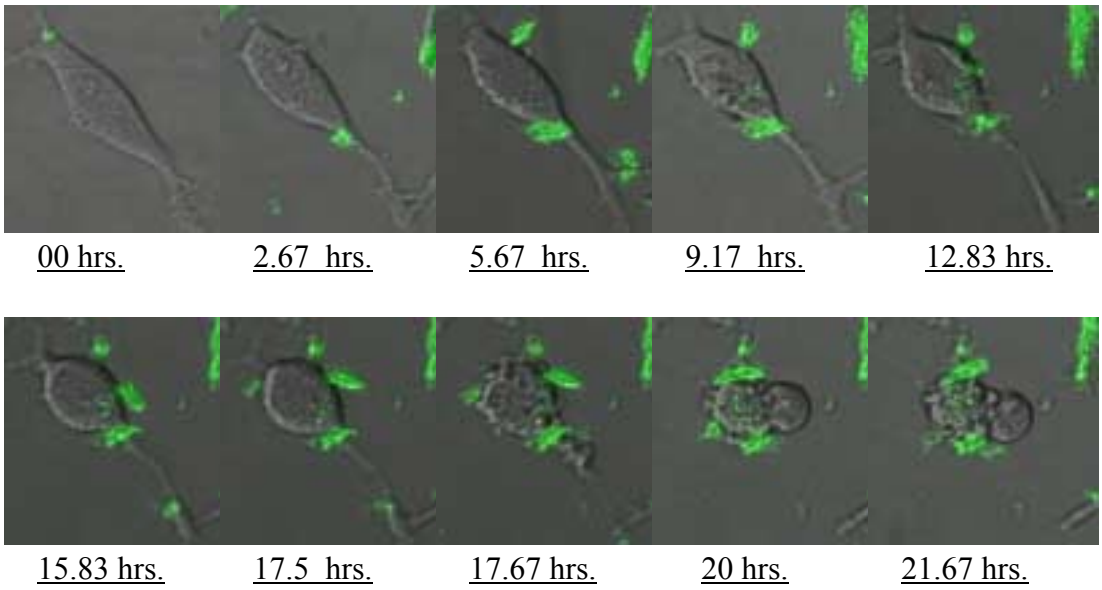


Figure 23. A live cell microscopic system monitoring a single cell treated with F-NLS-Q20 aggregates for 24hrs. PC-12 cells (NGF-differentiated) were incubated with F-NLS-Q20 aggregates for 24 hours and confocal images were acquired at various time points as indicated in the figure.

that the cell died at 17-20 hours, consistent with the LDH release assay time course study shown in Figure 22. Other cells in the field showed similar effects and a similar time course. The morphology of the cell death shown in the last 3 frames revealed cell blebbing and fragmentation, rather than swelling and leakage, suggesting that the cell death mechanism may be more related to an apoptotic pattern than a necrotic pattern. This will be further investigated in Chapter 6.

Discussion

Polyglutamine aggregates can be toxic to cells.

Debate continues as to whether it is the aggregated state of expanded polyglutamine sequences that is toxic to cells. The data presented by different groups are controversial. (See Chapter 1) The results described here clearly show that nuclear polyglutamine aggregates made *in vitro* are very toxic to mammalian cells, both PC-12 and Cos-7. The approach taken in our experimental system, of directly introducing polyglutamine aggregates into cells, is relatively clean and straight-forward. It eliminates some factors that made earlier experiments difficult to interpret, in allowing us to uncover a real toxic role of polyglutamine aggregates. First, it eliminates the possibility that monomer or aggregation intermediates, such as those that exist at least transiently in the transfected cell model systems, might be the toxic species. Second, technical factors may limit the ability to detect the formation of small aggregates in many cell studies. This was confirmed, in fact, by recent studies in our lab, in which an improved detection method allowed detection of a new type of recruitment-competent aggregate in transfected cells

and in HD brain tissues that cannot be detected by conventional methods (A. Osmand, V. Berthlier, E. Johnson and R. Wetzel, unpublished). In the work described here, we challenge the cells with exogenous aggregates so that any toxic effects can be clearly attributed to aggregates. Clearly, polyglutamine aggregates can be toxic.

The cytotoxicity of polyglutamine aggregates is linked to their nuclear localization

In our model system, aggregates of polyglutamine peptides localized to the nucleus of the cell are highly toxic, which is consistent with the results from transfected cell and transgenic animal experiments, in which expanded polyglutamine containing proteins targeted to the nucleus (also by NLS) produce nuclear aggregates and are highly toxic (Klement, Skinner et al. 1998; Saudou, Finkbeiner et al. 1998; Peters, Nucifora et al. 1999). This requirement for nuclear localization has been implicated by a number of studies, as described in Chapter 1, including gene transcriptional disruption. In addition, the results described here suggest that polyglutamine aggregates play a direct role in nuclear toxicity, which is not as clear in the expressed protein systems, since there are both monomers and intermediates present in addition to aggregates.

Our results show that aggregates localized to the cytoplasm, including perinuclear inclusions, exhibit little or no cytotoxicity. However, the more common observation of cytoplasmic aggregates in expanded CAG repeat disease human tissue (Gutekunst, Norflus et al. 2002), suggests a possible role for cytoplasmic aggregates in long-term toxicity, linked to neuronal dysfunction (see Chapter 1) rather than cell death. Further studies using our model system might be conducted to examine more subtle effects of

cytoplasmic polyglutamine aggregates on cell dysfunction. An alternative explanation for the presence of cytoplasmic aggregates in disease tissues is that cytoplasmic aggregates are, in fact, benign, but that - by mechanisms as yet unknown - cytoplasmic aggregates occasionally migrate into the nucleus, with cataclysmic results. In any event, in the context of our model system, the results clearly indicate that the toxic effects of polyglutamine aggregates follow upon their movement from the cytoplasm to the nucleoplasm.

The data supports a recruitment mechanism of cytotoxicity

As for the question of why nuclear aggregates are toxic, the results described up to this point do not provide conclusive evidence for any mechanism. However, together with the results of in *vitro* studies on the aggregation kinetics of these polyglutamine peptides and features of the aggregates they generate (Chen, Berthelier et al. 2001), this cytotoxicity study is consistent with a recruitment mechanism of polyglutamine aggregate toxicity. According to this mechanism, polyglutamine aggregates specifically recruit, sequester, and alter the activities of polyglutamine-containing proteins through a continuation of the aggregation process (explained in detail in chapter 1). Our results are consistent because (a) toxicity is only evident in the nucleus, where a lot of gene transcriptional factors and nuclear proteins have been identified that contain polyglutamine or glutamine-rich sequences; (b) aggregates of the control peptide, F-NLS-CspB-1, are efficiently introduced into the nuclei of the cells, but produce no toxicity. This is consistent with the recruitment mechanism, since these non-polyglutamine aggregates are expected to be inefficient in recruiting polyglutamine peptides, but would

be expected to be equally toxic to polyglutamine aggregates according to other mechanisms proposed for aggregate induced toxicity (reviewed in chapter1). From this point of view, it is possible that the very large, easily visible super-aggregates known as inclusions (NIIs) may be protective to cells, to the extent that they consolidate, and thereby reduce the effective surface area (Chen, Berthelier et al. 2001) of smaller, toxic aggregates.

Even though the present study was not designed to directly address other hypotheses and models of polyglutamine diseases, these experiments suggest that it is unlikely that the toxicity of polyglutamine aggregates is due to the saturation or inactivation of chaperones, proteasomes, or other cellular machinery by accumulated aggregates, which is an alternative hypothesis (Bates 2002). If that were the disease mechanism, it might be expected that nuclear Csp-B1 aggregates would be as toxic as polyglutamine aggregates in our experiments.

There is growing speculation that relatively small assembly intermediates may play key cytotoxic roles in a number of protein aggregation-related neurodegenerative diseases. Amyloid assembly intermediates of a number of proteins, including proteins not associated with disease, have been shown to have cytotoxic properties in cellular models. The identification of annular assembly intermediates in early aggregation timepoints in the amyloid formation of Aß, α-synuclein and other proteins has drawn increasing support for early speculations that amyloid-related toxicity is mediated by membrane insertion and depolarization.

The toxicity reported in this paper appears to be fundamentally different from the above examples, however, in a number of ways. First, the toxicity of our aggregates requires the presence of a polyglutamine sequence, while amyloid-like aggregates of a similarly treated and targeted control peptide are benign. Second, the toxicity of our aggregates, in parallel with toxicity in other cell models of HD, requires nuclear localization, whereas the target of the hypothetical membrane depolarizing assembly intermediates prepared from other amyloidogenic proteins is presumably the outer membrane. Third, the polyglutamine aggregates used here appear to be mature products of aggregation, rather than metastable assembly intermediates. While it is an attractive model that all protein aggregates kill cells by a common mechanism, the weight of evidence to date is that polyglutamine aggregate toxicity is mediated by a set of protein–protein interactions that are very specific to the expanded CAG-repeat diseases. Studies described in this chapter provide further support of a recruitment mechanism of toxicity.

Polyglutamine length cutoff in the disease mechanism is associated with efficiency of aggregate formation

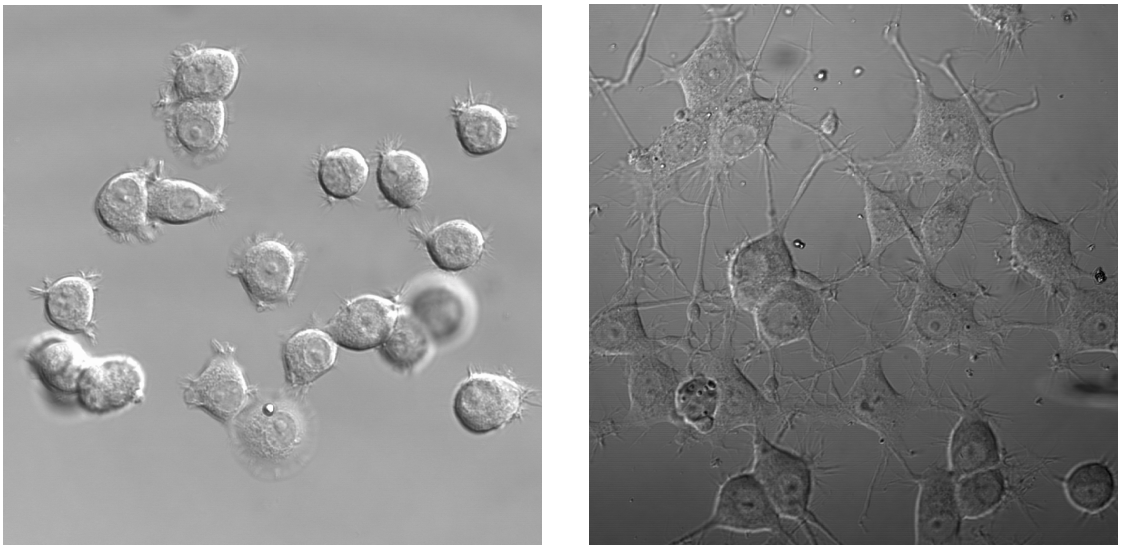
We show here that nuclear-localized aggregates of polyglutamine peptides are toxic regardless of peptide length. This observation supports the hypothesis that the repeat length dependence of disease risk in expanded CAG repeat diseases is related to the length dependence of aggregation efficiency. Once aggregates are formed, as governed by the biophysics of polyglutamine aggregation, and once these aggregates appear in the nucleus, any polyglutamine peptide aggregate is toxic. The simplest explanation for the link between polyglutamine repeat length and disease risk and age-of-onset is that

expanded polyglutamine sequences kill cells by virtue of their ability to efficiently form toxic aggregates.

No cell specificity observed in the system

Expanded polyglutamine proteins such as huntingtin are expressed in many cells throughout the body, and polyglutamine inclusions are observed in many brain regions in patient material and animal models (Reddy, Charles et al. 1999). In spite of this, only a small subset of neurons in highly localized regions are affected by these diseases. This data seems inconsistent with our data showing that Cos-7 and PC-12 cells have equal sensitivity to intranuclear polyglutamine aggregates. In addition, experiments using either NGF differentiated PC-12 cells or undifferentiated PC-12 cells show roughly the same effect. Figure 24A shows a phase-contrast microscopy image of the PC-12 cells with/without NGF induced differentiation and Figure 24B is a cytotoxicity study on the different aggregates using these differentiated cells. As can be seen from the Figure 24A, the morphologies between the differentiated and undifferentiated PC-12 cells differ dramatically; however, the toxicity induced by the aggregates (Figure 24B) remains the same. In fact, Cos-7 cells are also susceptible to toxic effects in cellular models involving by over-expressing of expanded CAG repeat genes (Abdullah, Trifiro et al. 1998; Merry, Kobayashi et al. 1998). This is probably the experimental limitation in all the cell models, in which the doses of expressed polyglutamine proteins (or the preformed aggregates, in our case) far exceed the intrinsic expression level of the proteins *in vivo*. At the experimental conditions of our experiment, we did not observe any specificity of cytotoxicity in different cells.

A PC-12 cells no NGF PC-12 cells with 50ng/ml NGF for 48hours



B

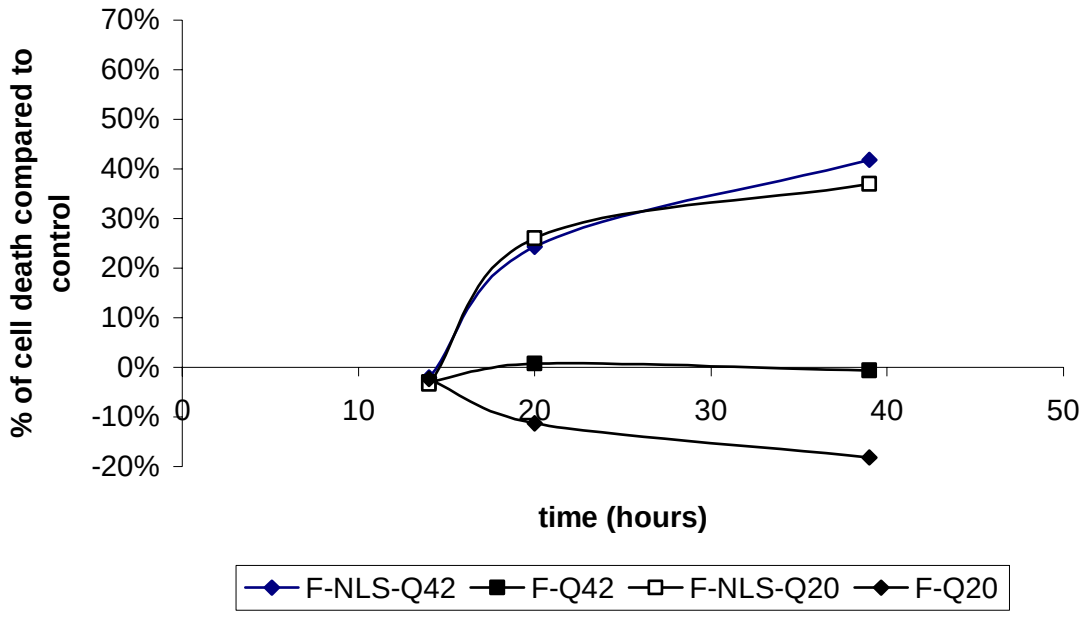


Figure 24. NGF differentiated PC-12 cells and the LDH assay for their cytotoxicity studies.

Experimental factors that affect the results

In the cytotoxicity experiments, there are conditions that affect the results with respect to both the cells and the aggregates. As discussed in Chapter 3, the limitation of the introducing aggregates into cells makes it difficult to explain some of the variations we observe with subtle changes in the experimental conditions. On the other hand, this negative information may also help us better understand the mechanisms at work.

For example, we observed that cells treated with nuclear targeted aggregates made at 37 °C (rather than frozen concentration) exhibit a limited cell death response. EM studies show that the morphologies of these two types of aggregates differ substantially (Chen, Berthelier et al. 2002). Furthermore, the abilities of the two types of aggregates to further recruit monomeric polyglutamine peptides (i.e., undergo extension) are also different, with the 37 °C aggregates being much less active than the -20 °C aggregates (Chen, Berthelier et al. 2001). This may help explain their different toxic effects. However, it cannot be ruled out that the toxic difference is also influenced by a decreased efficiency of cell uptake for the 37 °C aggregates. Similar to the above observations, there are conditions, like a requirement for ultracentrifugation of the soluble, monomeric peptide before the aggregation step, and sonication time after aggregation, that also affect the cytotoxicity results and which may have implications for the cytotoxicity mechanisms. Figure 25 is a diagram illustrating different ways in which aggregates of F-NLS-Q42 peptides were prepared, and Figure 26 shows a summary of cytotoxicity studies on these aggregates.

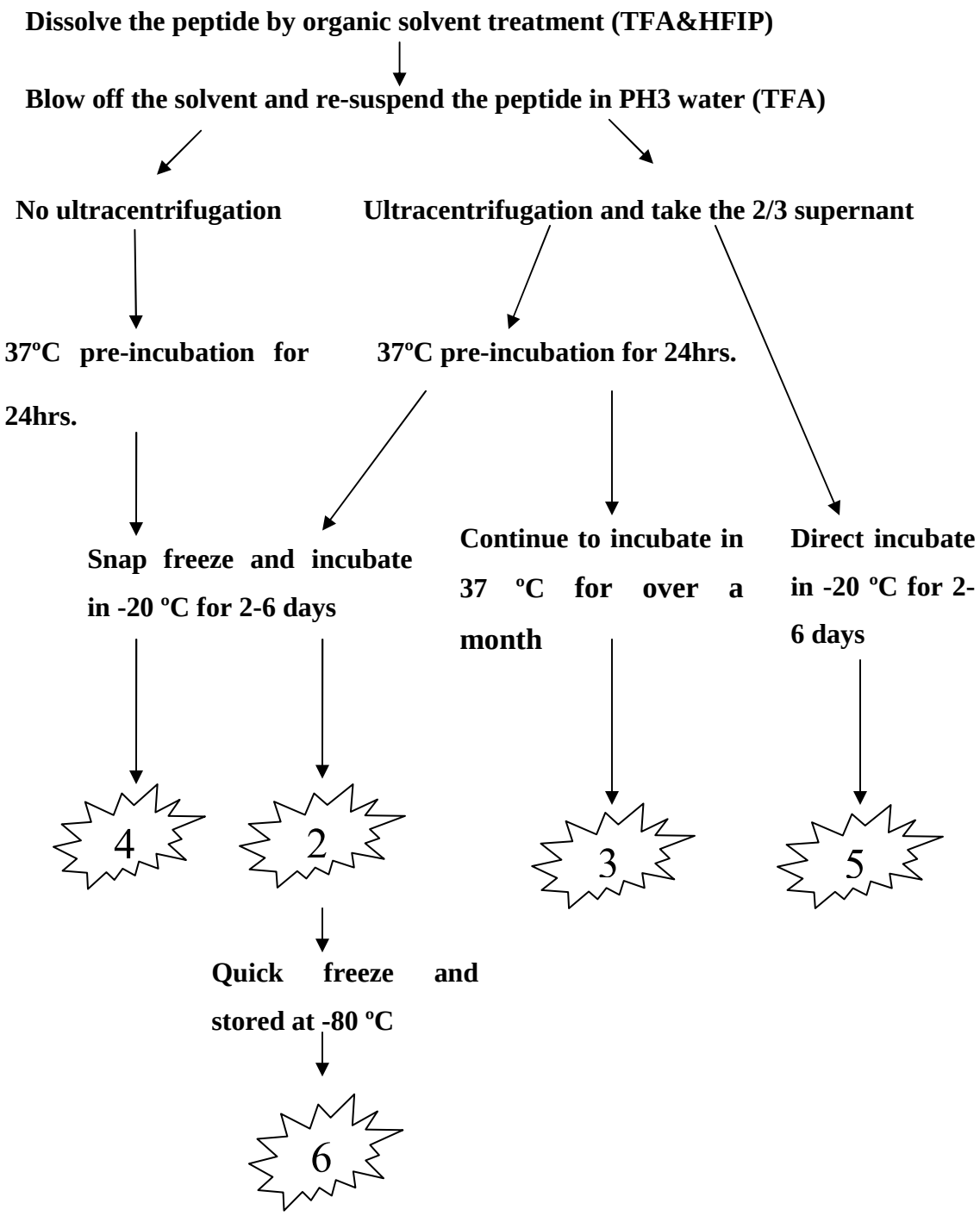


Figure 25. A diagram indicates the possible variants of the aggregates generated by the same peptide.

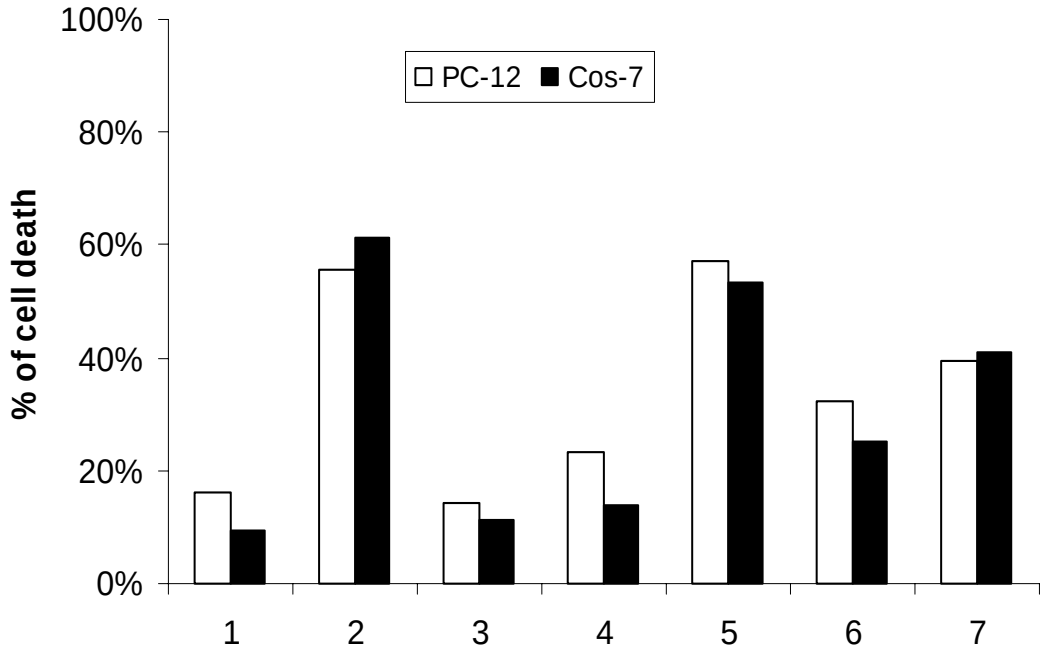
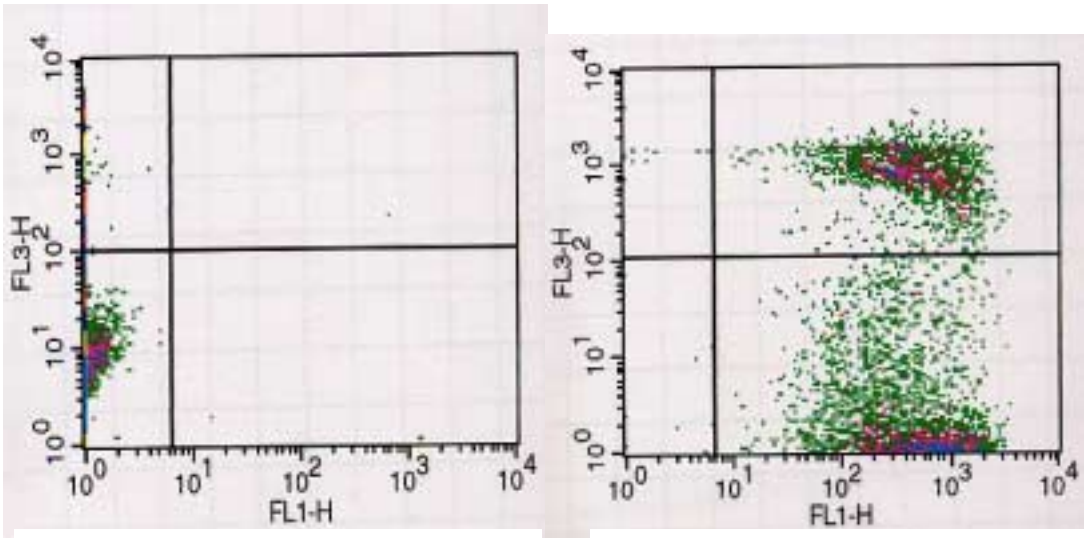


Figure 26. Cytotoxicity studies by LDH release assay on the experimental variants of aggregates of the F-NLS-Q42 peptides. The numbers is corresponding to aggregates showed in the previous diagram. In addition, 1 is a control; 6 is the storage of frozen aggregates after sonication and filtration at -80 ºC; 7 is No.6 with additional sonication and filtration.

We also observed that, even though there is no specificity between PC-12 and Cos-7 cells, the age and history of the PC-12 cells significantly affects their response to treatment with nuclear-targeted aggregates. That is, old cells (after passage over 15-20 times after receiving from stocks from UCLA) respond poorly to nuclear aggregates, exhibiting little or no cell death when treated with NLS-polyglutamine aggregates. Figure 27 is a flow cytometry study using aged PC-12 cells (passage 24), which showed essentially no toxicity for F-NLS-Q42 aggregates that, on "young" (3-5 passages) PC-12 cells, showed high toxicity (Figure 15-16). There are at least two possible reasons: (a) old cells may have reduced ability to take up aggregates or to deliver them into nuclei; (b) the transcriptional factors (hypothetically) affected by NLS-polyglutamine aggregates may not be present or essential for older cells to survive. It is well-known that cultured cancer cell lines tend to grow more and more rapidly and require less and less growth support (serum or specific growth factors), suggesting that aged cells in culture may simplify the pathways required for viability. This has been reported in other cell lines (Hamelers, van Schaik et al. 2002).

There may be additional parameters that also affect the reproducibility of these experiments that we did not notice and explore. For this reason, it is very important to follow the described protocols exactly in order to obtain similar results.



(-) control: 25.49% cell death **F-NLS-Q42 treated: 23.55% cell death**

Figure 27. Cytoxicity study on aged PC-12 cells (24 passages) by flow cytometry measured PI uptaking.

CHAPTER 5

TOXICITY OF SOLUBLE POLYGLUTAMINE PEPTIDES

Cytotoxicity study on soluble polyglutamine peptides using the LDH release assay

Dose-dependent cytotoxicity of soluble polyglutamine peptides

To make our studies more complete, we also carried out some experiments with soluble polyglutamine peptides. Figure 28 shows the results of a dose-response study initiated by applying monomeric peptides to PC-12 cells in culture. All of the experimented peptides are non-toxic up to 100 uM, with the single exception of monomeric F-NLS-Q_{42}. The toxicity observed for monomeric F-NLS-Q_{42} is almost certainly due to its strong ability to rapidly aggregate under the experimental conditions. In fact, at the end of the incubation period, prior to LDH-release analysis, at toxic concentrations of the Q_{42} peptide, aggregates are clearly visible surrounding and covering the cells, and fluorescent foci are observed in the cells by confocal microscopy as shown in Figure 30. The lack of toxicity observed for monomeric F-NLS-Q_{20}, in contrast to the toxicity observed for the aggregated version, again emphasizes that aggregation is required for cytotoxicity.

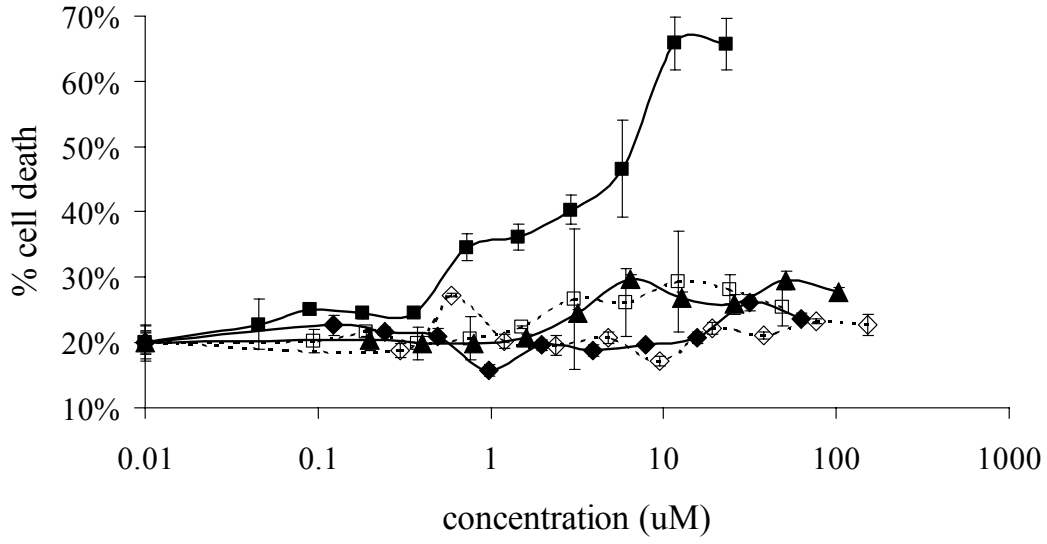


Figure 28. Dose-dependent cytotoxicity of polyglutamine monomers. PC-12 cells were incubated with various concentrations of polyglutamine monomers and cell death measured by the LDH-release assay. F-NLS-Q_{42}, closed squares (■); F-Q_{42}, open squares (□); F-NLS-Q_{20}, closed diamonds (◆); F-Q_{20}, open diamond (◇); F-NLS-CspB-1, closed triangles (▲). The data reflects the mean values of 2-4 independent repetitions, each conducted in triplicate; data was normalized to a value of 20% cell death for the untreated cells. The average EC_{50} for monomeric F-NLS-Q_{42} is about 4-5 uM, while monomeric F-NLS-Q_{20} was not toxic up to 100 uM.

Time course of the cytotoxicity by soluble polyglutamine peptides

The time course of cell death in this monomer study shows that the only monomeric polyglutamine peptide to exhibit toxicity, presumably by virtue of contemporaneous aggregation, is F-NLS-Q_{42} (Figure 29). The greater extent of cell killing in experiments initiated with monomeric F-NLS-Q_{42} is possibly due to the ability of smaller, nascent aggregates to better penetrate cell and nuclear membranes.

Monitoring a single cell for monomer toxicity

We also did parallel live cell experiments using solublized F-NLS-Q_{42} peptides, in which we attempted to visualize the dynamic structural changes occurring in the cells taking up monomer, and the process of monomer aggregation. Figure 30 shows selected pictures of a single cell in a 24 hr time course. The data suggests that (a) aggregates appear both in and outside the cell; and (b) cell die in the 20-22 hr time frame when there is nuclear localization of aggregates. This data is consistent with the time course study measured by the LDH release assay shown in Figure 29.

Discussion

Stronger evidence for the toxicity of aggregates

The results using the monomeric form of polyglutamine peptides speaks against disease mechanisms that invoke unique toxic features of special conformations of

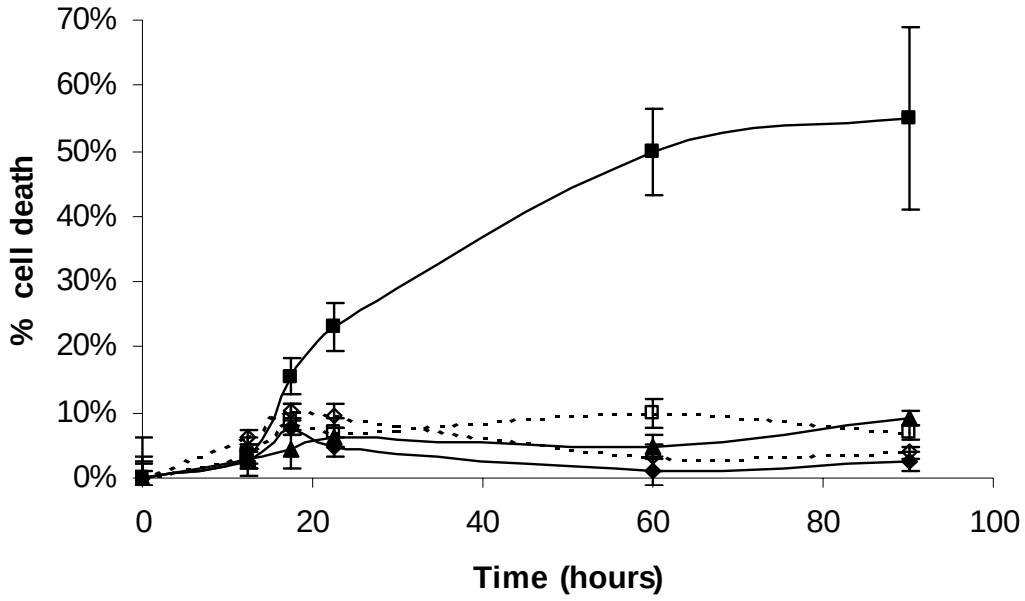


Figure 29. Time course of the cytotoxicity of polyglutamine monomers. PC-12 cells were incubated with polyglutamine aggregates (A) or monomers (B) for over 90hours and cell death was measured by the LDH-release assay at various time points. F-NLS-Q_{42}, closed squares (■); F-Q_{42}, open squares (□); F-NLS-Q_{20}, closed diamonds (◆); F-Q_{20}, open diamond (◇); F-NLS-CspB-1, closed triangles (▲). Two independent experiments were conducted, each in triplicate for each. Data was normalized by subtracting out the control at each time point; the percent cell death is based on the increased cell death compared to control.

F-NLS-Q42 Monomer treated

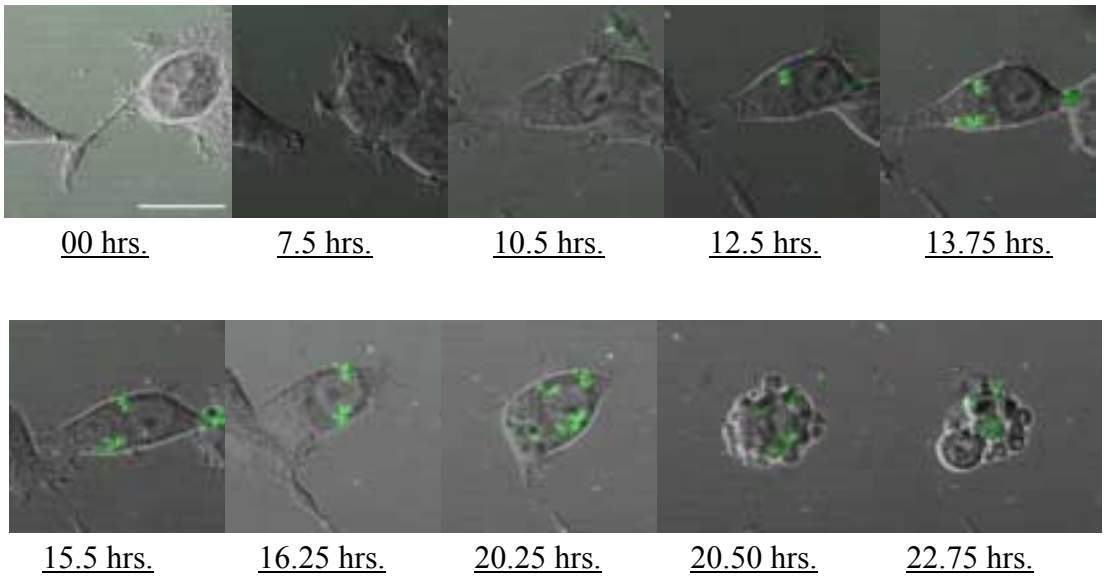


Figure 30. A live cell microscopic system monitoring a single cell treated with F-NLS-Q42 monomers for 24hrs. PC-12 cells (NGF-differentiated) were incubated with F-NLS-Q_{42} aggregates for 24 hours and confocal images were acquired at various time points as indicated in the figure.

monomeric forms of expanded polyglutamine repeat proteins. CD data suggests that both monomeric Q_{20} and Q_{45} contain the same structure, random coil (Chen, Ferrone et al. 2002). However we observed that, for Q_{20}, only the aggregated form is toxic. It is interesting that the time course for cytotoxicity is very similar for both the aggregates and monomer experiments (for Q_{42}). However, it is not clear from our experiments whether monomeric peptides enter cells first and then aggregates intracellularly, or whether aggregates formed outside of the cells enter cells by the normal mechanism. It is worth nothing that in this experiment, the aggregates grown at 37 °C, suggesting that there is no intrinsic difference in the toxic morphology of 37 °C aggregates compared to -20°C aggregates.

Limitations

Using monomer to do these experiments might be viewed as a regressive step, taking us back into the pool of debating whether monomers or aggregates are responsible for cytotoxicity. With the availability of good transfected cell model systems, the advantage of using monomer seems weak. It is also very hard to monitor monomer inside the cell, since the fluorescence from the monomeric forms of the peptides is very faint under the confocal microscope and only is visible after they form aggregates. One advantage of using exogenous peptides over transfected cells is that new sequences can be tried without need to construct a new vector. Our experiments with monomer were conducted mostly as a control for the aggregate experiments. Nevertheless, there may be situations in which a model involving the treatment of cells with monomer might be advantageous.

CHAPTER 6

MECHANISMS OF POLYGLUTAMINE AGGREGATE-ASSOCIATED CELL DEATH

A requirement for new protein synthesis

As reviewed in Chapter 1, cell death can be divided into two major groups, programmed (mainly apoptosis) and non-programmed necrotic cell death. Thus, the first step in understanding the mechanism of cell death observed in nuclear polyglutamine aggregate treated cells is to assign one of these broad classifications. We used protein synthesis inhibitors to find out whether or not the cell death in our system requires new gene transcription and translation for cell death to occur. Actinomycin D (Act. D) is an anti-neoplastic antibiotic. It inhibits RNA polymerase activity by blocking its binding site to DNA and therefore inhibits gene transcription and stops new protein synthesis. Cycloheximide (CHX) is an antibiotic produced by *S. griseus*. Its main biological activity is inhibition of protein translation in eukaryotes resulting in inhibition of protein synthesis. It has been shown that both Act.D and CHX can act as inhibitors for programmed cell death (Sperandio, de Belle et al. 2000). In Figure 31, we treated PC-12 cells with 1.0 ug/ml Actinomycin D or cycloheximide 4 hours after incubation with nuclear aggregates. The results in Figure 31 show that increased cell death induced by nuclear aggregates is diminished by addition of both types of protein synthesis inhibitor. This data indicates that protein synthesis is required for the cell death induced by nuclear

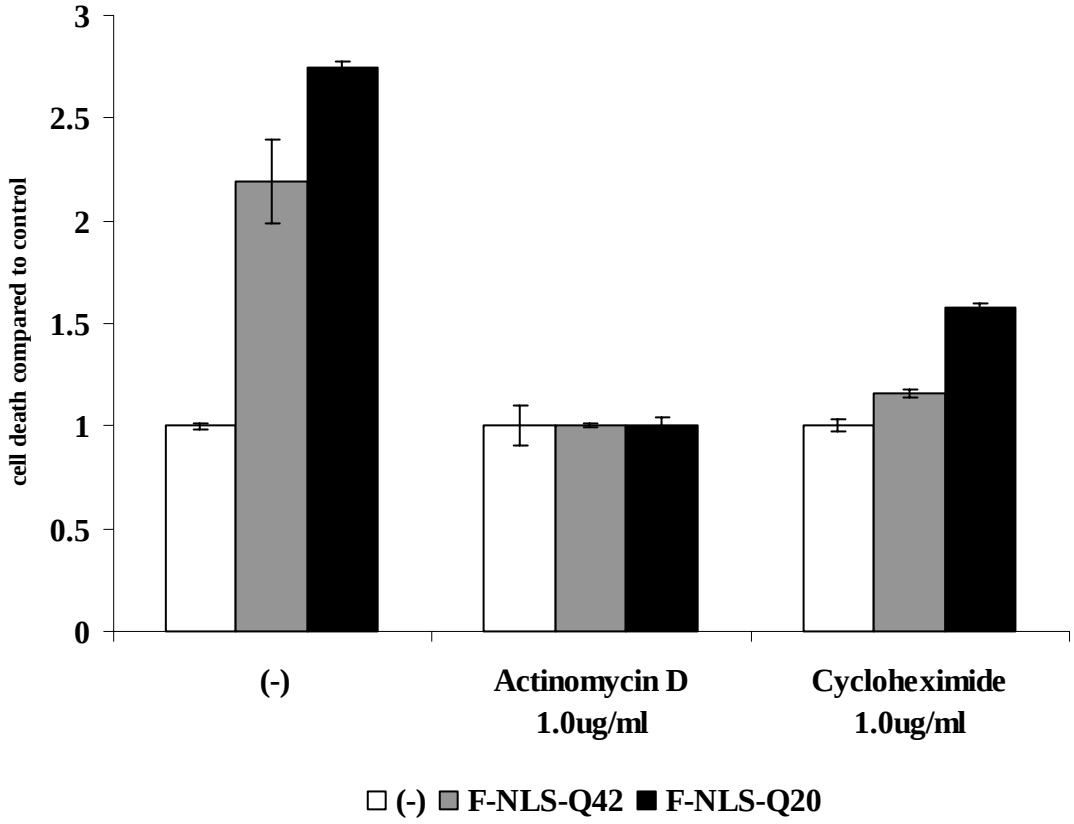


Figure 31. Protein synthesis inhibitor studies. PC-12 cells were treated with/without nuclear aggregates (F-NLS-Q_{42} or FNLSQ_{20}) for 4 hours before adding actinomycin D or cycloheximide at 1.0 ug/ml, after which cells were incubated an additional 16 hours. The LDH assay was used to evaluate the degree of cell death. The data is presented by comparing cell death to the control in each group (cell death compared to control = % of cell death induced by aggregated / % of cell death in the control without aggregate treatment).

aggregates. The normal interpretation of such data is that these new proteins must be ones required for the suicide cell death cascade; in contrast, simple necrosis should not require new protein synthesis. Thus, this test suggests that the cell death in our system is a programmed cell death, probably apoptosis.

A requirement for caspase activation for cell death

PARP cleavage implicates caspase activation

Other tests for apoptotic mechanisms inquired whether caspase activation is required for cell death (Chapter 1). We first used a western blot approach to detect a common substrate of caspase, Poly (ADP-ribose) polymerase (PARP). Specific cleavage of PARP from 116 kD to 85 kD fragment is frequently used to test for caspase activation (Lazebnik, Kaufmann et al. 1994). Thus, PARP (Figure 32) is cleaved to an 85 kD fragment when cells are incubated with stauroporine, which has been shown to induce apoptosis in a number of studies (Li, Cheng et al. 1999). Figure 32 also shows that cells treated with NLS polyglutamine aggregates, but not with other aggregates, exhibit significant PARP cleavage. This data suggests that caspases are activated during the cell death process, implicating an apoptotic mechanism.

Caspase inhibitor study also implicates caspase activation

We also carried out a second, indirect assay for the role of caspase activation by using a universal caspase activation inhibitor Z-VAD-FMK to see whether or not we can rescue the cell death induced by aggregates. Figure 33 shows an experiment, in which we

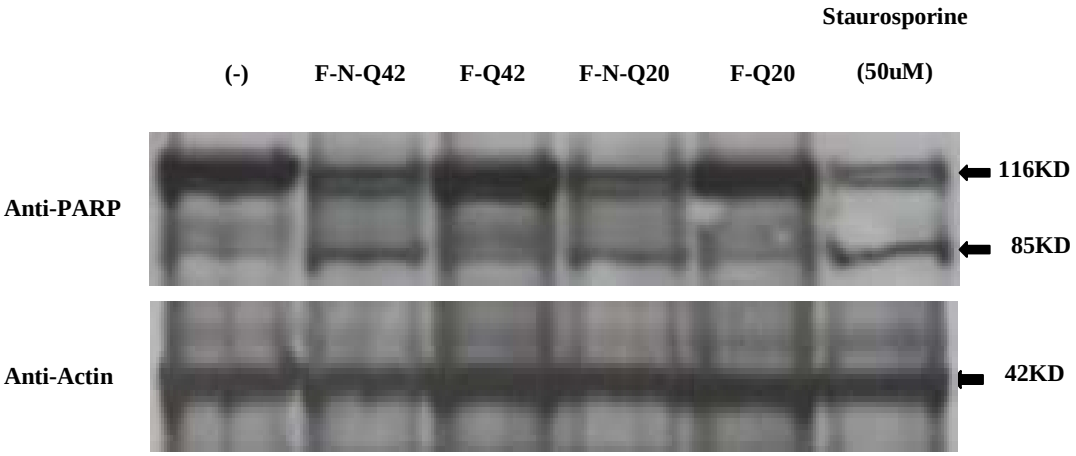


Figure 32. Western blot for PARP cleavage. PC-12 cells were incubated with/without different aggregates as indicated in the figure for 36 hours. In the positive control for apoptosis, cells are treated with staurosporine at 50 uM for 24 hours. The anti-actin band shows that equivalent amount of cellular extract were loaded in each lane of the gel.

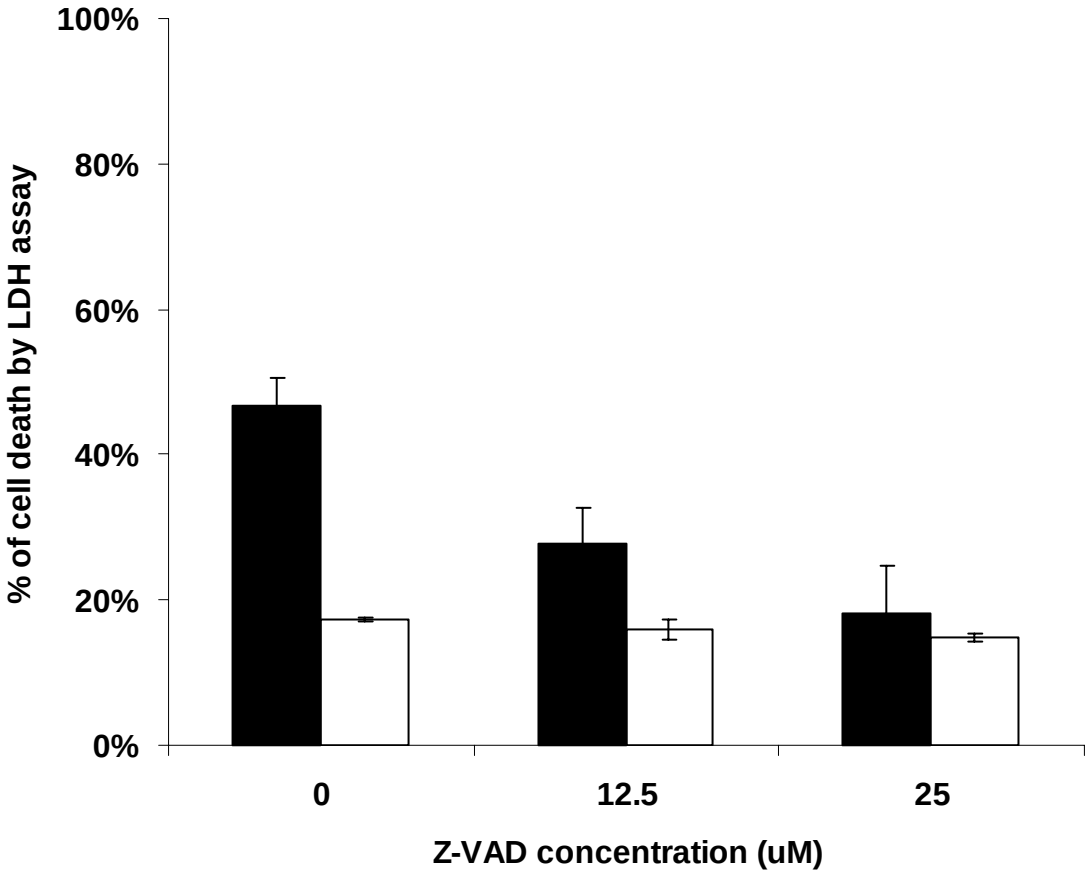


Figure 33. Caspase inhibitor (Z-VAD-FMK) study. PC-12 cells were co-incubated with F-NLS-Q_{42} aggregates (filled bars) or PBS (open bars) and with Z-VAD-FMK at different concentrations, as indicated, for 20 hours. The LDH assay was use to evaluate cell death.

co-incubated the cells with both the F-NLS-Q42 aggregates and Z-VAD-FMK for 20 hours. The result is that the cell death induced by the nuclear aggregates is dramatically decreased in the presence of caspase inhibitor compared to control without inhibitors. Figure 33 also shows that the cell rescue by the caspase inhibitor exhibits a dose-dependent effect. This data clearly shows that caspase activation is required for the cell death, although the identity of the caspases involved is not revealed. The PARP cleavage and caspase inhibitor results are in agreement with the protein synthesis inhibitor results in implicating an apoptotic mechanism for polyglutamine aggregate induced cell death.

DNA fragmentation and Annexin-V conjugation assay

To further confirm the possible apoptotic cell death mechanism, we attempted to characterize aggregated killed cells by the biochemical hallmark of apoptosis, internucleosome DNA fragmentation. This was carried out in two approaches, agarose gel electrophoresis of DNA laddering (a serial fragmented DNA with 180-200 bp increments) and a sensitive ELISA assay detecting the fragmented nucleosomes.

DNA laddering is a classic apoptosis measurement, often used to distinguish apoptosis from necrosis (which gives a smear of DNA with no ladder pattern). Figure 34 shows the results of a laddering experiment, including a control DNA ladder provided by the manufacture. Unfortunately, none of the experimental lanes show any DNA fragmentation, either ladder or smear (Figure 34). We also used a more sensitive ELISA assay purchased from Roche Applied Science, which quantitatively measures the fragmented DNA by a photometric enzyme-immunoassay approach (illustrated in Figure 35). The experiment worked perfectly in a positive control sample (stauroporine treated

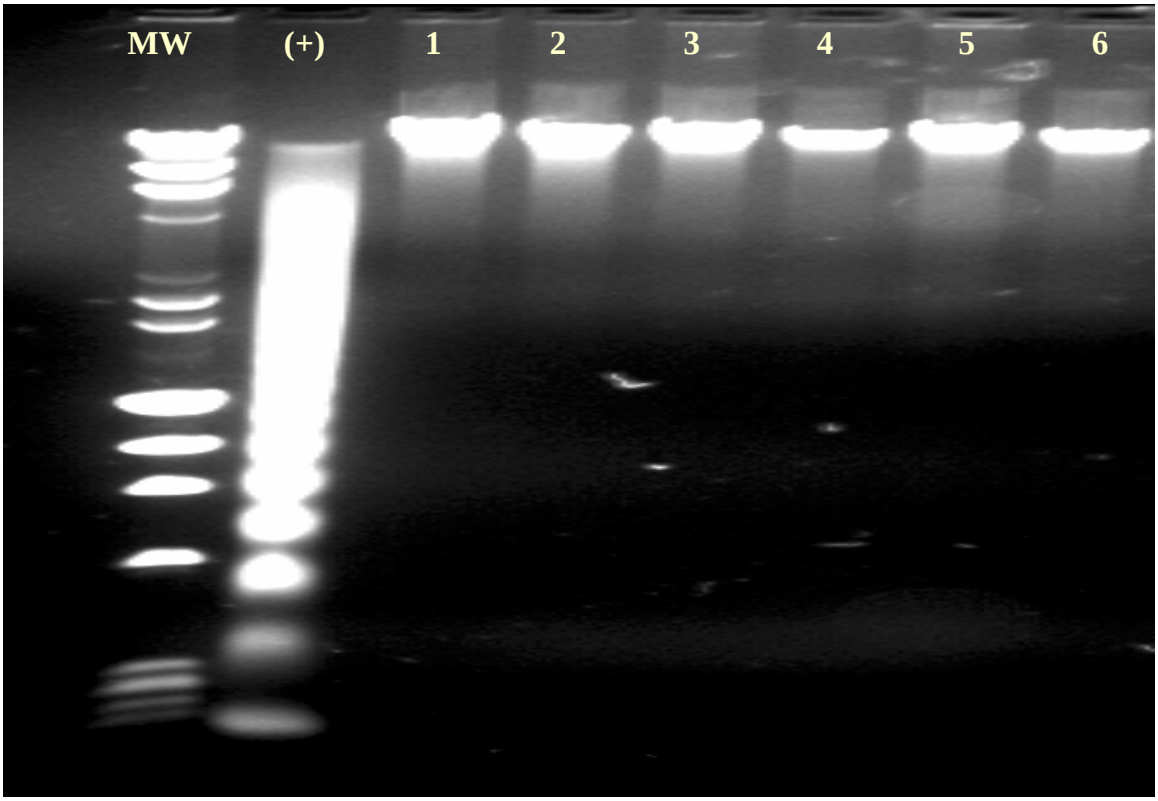


Figure 34. An agarose gel monitoring DNA fragmentation in cells treated with aggregates for 48 hours. (1) PC-12 cells (-) control; (2) F-NLS-Q_{42} aggregate treated PC-12 cells; (3) F-Q_{42} aggregate treated PC-12 cells; (4) Cos-7 cells (-) control; (5) F-NLS-Q_{42} aggregate treated Cos-7 cells; (6) F-Q_{42} aggregate treated Cos-7 cells.

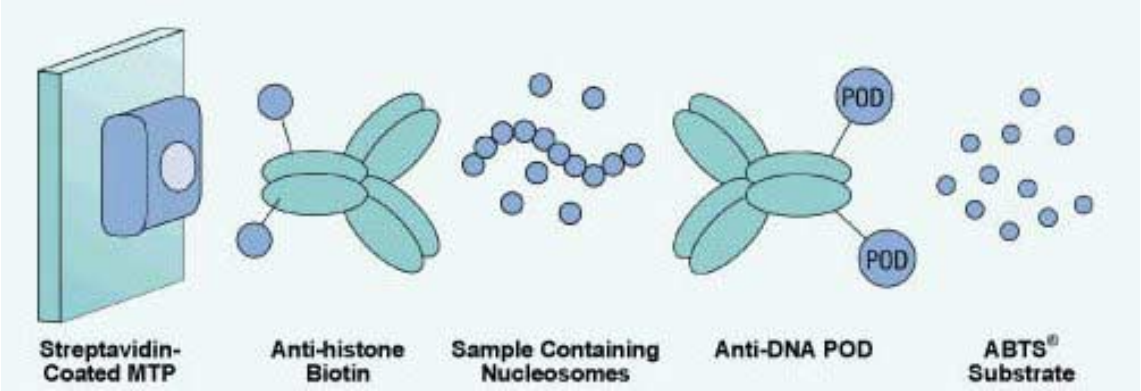


Figure 35. A diagram illustrating the principle of detecting fragmented DNA using ELISA assay (from Roche Applied Science). POD is a peroxidase conjugate that react with ABTS substrate and produce a color that can be measured at OD 405 nm.

PC-12 cells), but none of the aggregate treated PC-12 cells showed DNA fragmentation using this method (Figure 36). As we clearly see cell death induced by nuclear polyglutamine aggregates by the other cell viability studies, it is hard to interpret these negative DNA fragmentation results with any known cell death models. One possible explanation for these negative results is that fragmented DNA (negatively charged) might bind to the aggregates (positively charged) and cause precipitation during the centrifugation step to for extracting DNA. In confirmation of this, Figure 36 (last lane) shows diminished DNA fragments after simply incubating the already fragmented DNA (after stauroporine treatment) with polyglutamine aggregates. Thus, DNA laddering might be occurring in polyglutamine aggregate-treated cells, but not be observable. In the future, in order to visualize DNA fragmentation, it maybe possible to modify the DNA extraction conditions or use other assays that do not involve centrifugation, such as an *in situ* TUNEL assay to avoid the precipitation of the fragmented DNA.

We also tried another assay widely used in apoptosis studies, the Annexin V binding assay. When cells undergo apoptosis, the membrane phosphatydylserine (PS), which is normally located on the cytoplasmic side of the cell membrane, is translocated and exposed on the cell surface. This can be detected using a human vascular anticoagulant, annexin V, which selectively binds to PS (van Engeland, Nieland et al. 1998). We used Annexin-V labeled with a fluorescent tag (purchased from Molecular Probes, Inc.), which can be assayed by microscopy or flow cytometry. Figure 37 shows a confocal microscopic image of F-NLS-Q_{42} aggregate-treated cells stained with tagged annexin V, indicating possible annexin staining. However, annexin V can also stain

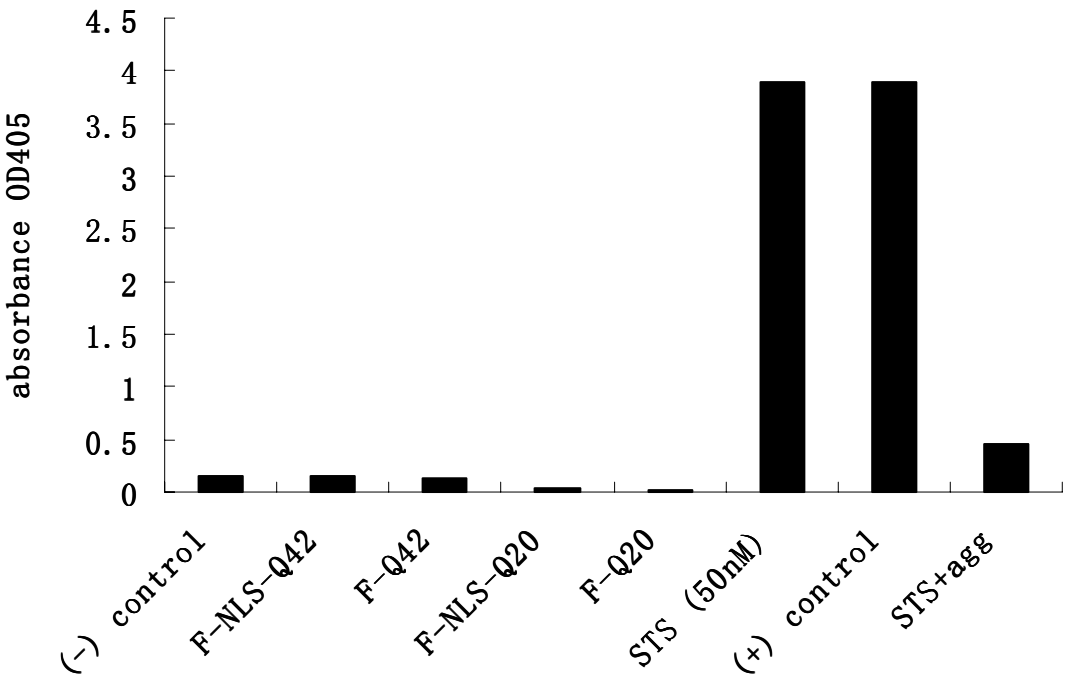
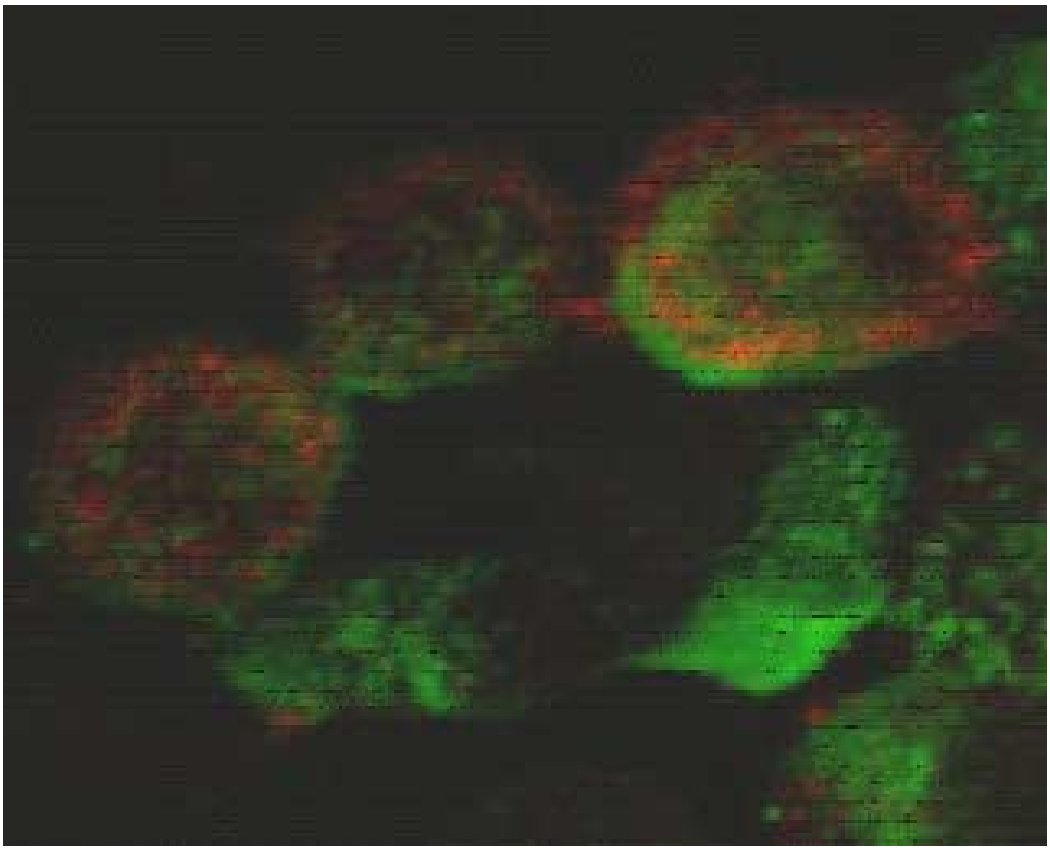


Figure 36. ELISA assay detecting DNA fragmentation. PC-12 cells were incubated with different aggregates for 22 hours. The cells were collected by centrifugation and lysed using mild detergent. The fragmented DNA was collected to perform the ELISA assay for detecting the fragmented DNA. (-) control is the cells with no aggregate treatment; Stauroporine (STS) treated cells are a positive controlfor apoptosis; Another positive control was a sample of fragmented DNA provided by the manufacture; the last lane is the supernant collected form incubation of F-NLS-Q42 aggregates with cells treated with STS for 48 hours, to test whether the aggregates could compromise the detection of DNA fragments.



F-NLS-Q42 aggregates treated cells

Figure 37. A confocal microscopic image of Annexin V binding of the PC-12 cells treated with F-NLS-Q42 aggregates. Aggregates are green and Alexa Fluor 647 conjugated annexin V is red in the figure.

necrotic cells, since after membrane integrity is lost annexin V can pass through the membrane and bind to PS resident on the inner surface. Thus, annexin labeling can only be rigorously interpreted using a third indicator, for membrane integrity (which is still intact in early apoptosis, but lost in necrosis or late apoptosis). PI staining for the nucleus upon loss of cell membrane integrity is normally interpreted as evidence for necrosis. It should be possible to detect early apoptosis (while the membrane is still intact) in the cells treated with the NLS-polyglutamine aggregates. This requires 3 different fluorescence channels (green for polyglutamine aggregates, red for PI, another color for Annexin V), which we is beyond the capabilities of our flow cytometry facility.

A summary of these cell death mechanism results, compared to the expected results for apoptosis and necrosis cell death mechanisms, is listed in Table 9.

Discussion

Mechanism of cell death induced by NLS-polyglutamine aggregates

Given the fact that the cell death in our model system requires protein synthesis and caspase activation, the mechanism of the cytotoxicity of nuclear polyglutamine aggregates in our system seems to be clearly an apoptosis-type mechanism. According to the recruitment hypothesis, nuclear aggregates recruit and sequester polyglutamine-containing proteins such as transcriptional factors, which then cause abnormal gene transcription or other effects. It is well-known that transcription dysregulation can become so severe that cells can be induced to undergo apoptosis (Cha 2000). Such an

Table 9. Comparison of the cell death induced by the nuclear polyglutamine aggregates with response typical of apoptosis and necrosis

	Apoptosis[a]	Necrosis[a]	Aggregate-treated cell death[b]
Annexin V binding	+	+	+
Internucleosomal DNA fragmentation:			
DNA laddering gel[c]	+	-	-
DNA fragmentation ELISA[c]	+	-	-
Caspase activity:			
PARP cleavage (118 kD to 85 kD)	+	-	+
Inhibition by:			
z-VAD (caspase inhibitor)	+	-	+
Protein synthesis inhibitor:			
Actinomycin D	sometimes	-	+
Cycloheximide	sometimes	-	+

[a] from the literature

[b] in all cases, cells were treated with aggregates of F-NLS-Q42

[c] experimental result compromised by direct aggregate-DNA interaction as described in text.

apoptotic mechanism, in the absence of phagocytes, will eventually cause the cell die completely, with development of some necrotic features such as membrane disruption. This presumably explains the ability of the LDH release and PI exclusion assays to monitor cell death in our model, in spite of its initiation by an apoptotic pathway (illustrated in Figure 38).

Further studies need to be carried out

So far, we have not directly approached the question of gene transcriptional dysregulation. A DNA microarray or differential display RT-PCR can be carried out in the future to study the changes in the profile of gene transcription in nuclear aggregate treated cells. This will be very valuable not only to understand this model system better, but also to compare data on our system with data already generated from the transfected model system.

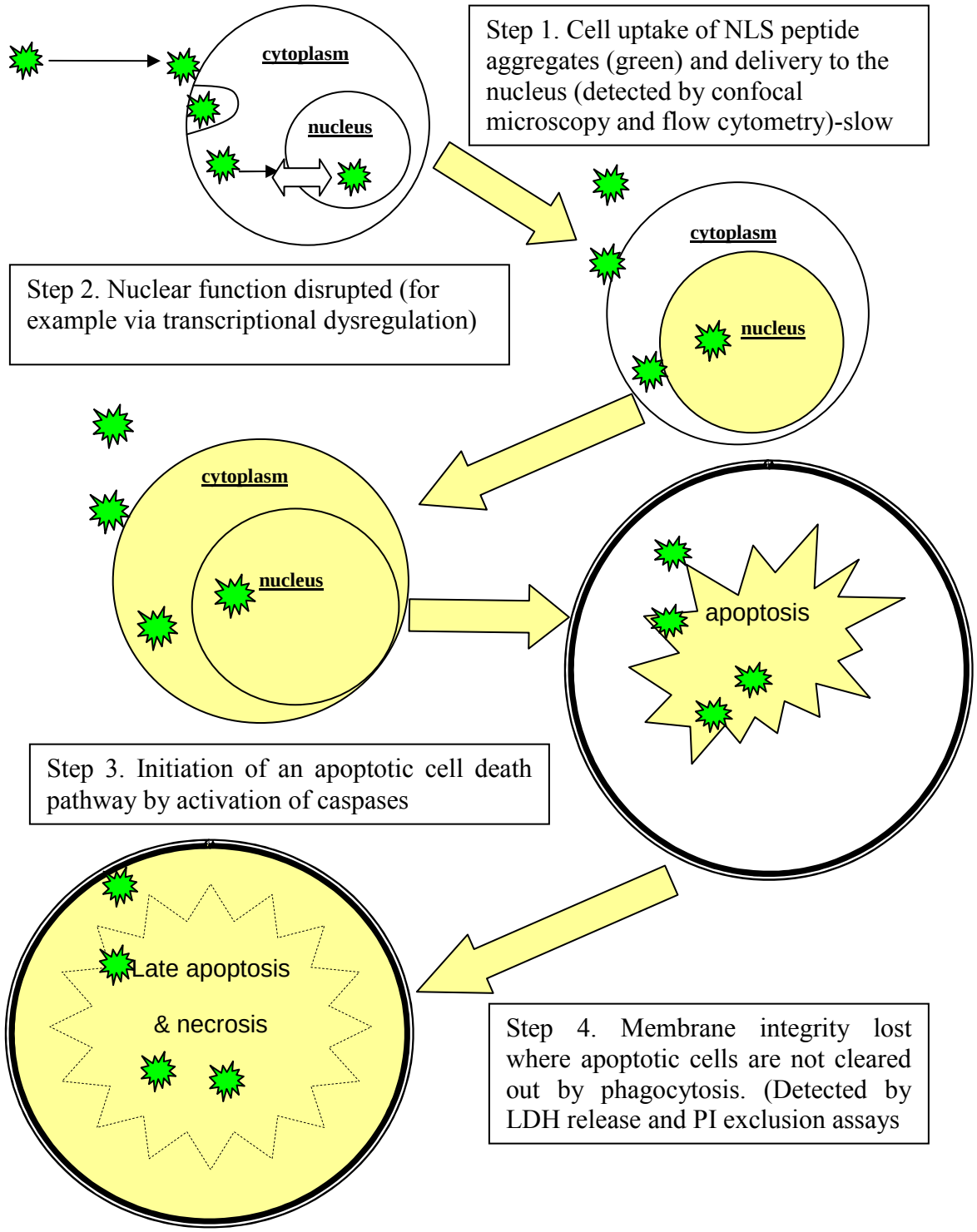


Figure 38. A diagram of the hypothesis on the events of cytotoxicity induced by nuclear polyglutamine aggregates.

CHAPTER 7

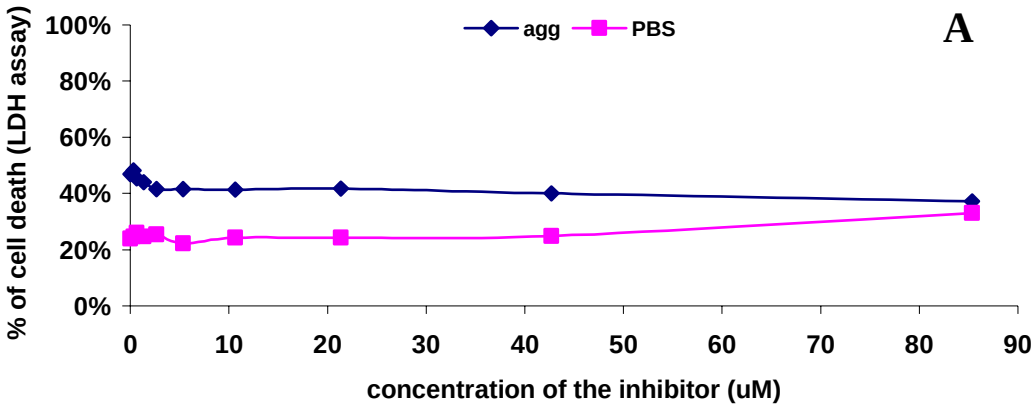
EFFECT OF A POLYGLUTAMINE ELONGATION INHIBITOR ON AGGREGATE-INDUCED CELL DEATH

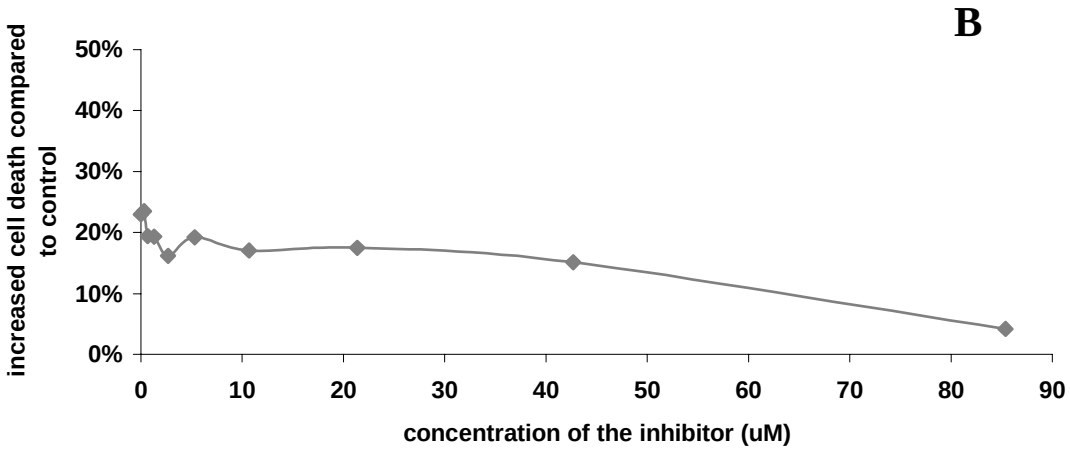
An elongation inhibitor protects cells from aggregate – associated cell death

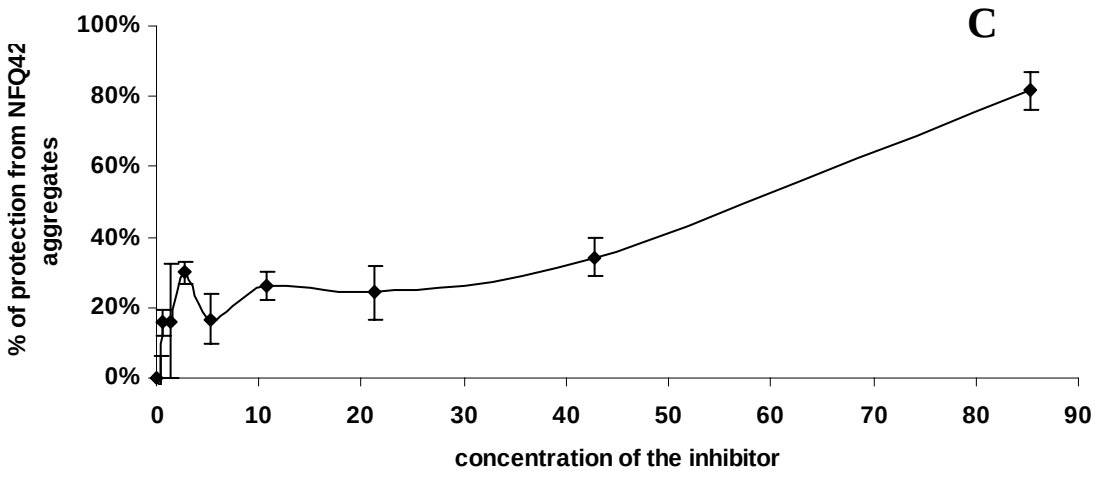
In a recent paper, Thakur and Wetzel (Thakur and Wetzel 2002) explored the structural organization of polyglutamine aggregates. By introducing Pro-Gly pairs (which has to be positioned at the turn position in a β-sheet) in the polyglutamine sequence, Thakur and Wetzel discovered a peptide named PGQ9, that aggregate in an identical fashion to a simple polyglutamine peptide of the same length, suggesting that PGQ9 probably adopts the same β-sheet structure as the polyglutamine sequence. To confirm this, they introduced additional prolines in positions thought to be essential for extended chain formation in the proposed β sheet structure. In fact, such peptides, one of which is $PGQ_9(P^{2,3})$, do not aggregate, confirming the structural hypothesis.

The complete amino acid sequence of $PGQ_9(P^{2,3})$ is listed in Figure 39. Subsequent studies on *in vitro* fibril elongation of normal polyglutamine aggregates done in the presence of this peptide revealed that, $PGQ_9(P^{2,3})$ can also inhibit this elongation. To test the hypothesis that the recruitment activity of an aggregate is important for its toxicity, we asked whether or not this recruitment inhibitor can prevent cell death in our model system. We used different amounts of the peptide from 0 uM up to 100 uM. The

Figure 39. Study of the protection effect of an elongation inhibitor on the cytotoxicity of F-NLS-Q42. PC-12 cells were pre-incubated with different doses of PGQ9($P^{2,3}$), as indicated in the figure, for 24 hours, then F-NLS-Q_{42} aggregates were used to treat the cells according to the usual method. A is the original data showing cell death measured by LDH release; B is this data corrected by subtracting out the PBS control; C is the data shown in B normalized by the equation: % of protection from F-NLS-Q42 aggregates = (increased cell death caused by the aggregates without the inhibitor – increased cell death caused by the aggregates with the inhibitor at each dose point) / increased cell death caused by the aggregates without the inhibitor.







$PGQ_9(P^{2,3})$: K_2-Q_9-PG-Q_4PQ_4-PG-Q_4PQ_4-PG-Q_9-K_2 46 amino acid

inhibitor peptide was preincubated with the cells for 24 hours, after which aggregates were incubated with the cells with the continued presence of the inhibitor. Figure 39A shows that the cell death induced by F-NLS-Q42 aggregates was significantly inhibited by the presence of the elongation inhibitor. By further processing the data by correcting for the direct cell toxicity of the inhibitor in the PBS control, Figure 39B shows that the cell death contributed solely by nuclear polyglutamine aggregates is decreased from 25% to 5%, as inhibitor concentration increases. Figure 39C presents the percentage of the cell death rescued by the inhibitor. The data reveal a significant, dose-dependent cell death rescue effect by the inhibitor. At 80 uM concentration of the inhibitor, up to 80% of the cell death induced by the nuclear aggregates was blocked. This rescuing effect is most probably due to the inhibition of the recruitment of other polyglutamine peptides, which greatly supports the recruitment mechanisms of toxicity of polyglutamine peptides.

Discussion

This data is the most compelling data available indicating that the polyglutamine-recruitment activity of aggregates is responsible for polyglutamine aggregate toxicity. It also suggests a possible drug discovery approach focusing on elongation inhibition.

It is still unclear as to how $PGQ_9(P^{2,3})$ works, since we haven't visually traced the peptide after incubating it with the cells. For example, the peptide may remain in the medium, where it binds to the aggregates and is subsequently delivered into the nucleus together with NLS-aggregates. Once there, it could prevent further recruitment of other polyglutamine containing transcriptional factors and rescue the cell. Alternatively, the peptide may be transported into the cells first. For a potential treatment using this peptide,

we need eventually to deliver the peptide into cells or even the nucleus. A this time, we cannot demonstrate whether or not the peptide was delivered into nucleus by incubating it with the cells. Further studies on the inhibitor peptide thus need to be carried out.

CHAPTER 8

CONCLUSIONS

In conclusion, we have developed a new cell model system to study the mechanisms of polyglutamine aggregate toxicity.

The results described in this dissertation suggest that polyglutamine aggregates made *in vitro* can be efficiently delivered into mammalian cells, both PC-12 cells and Cos-7 cells. Facilitated by NLS sequences, aggregates can be delivered to the nuclei of the cells. The rapid cytotoxicity of polyglutamine sequences requires both their aggregation and their nuclear localization.

In our model system, aggregates of polyglutamine peptides localized to the cytoplasm, including perinuclear inclusions, are not toxic. Likewise, aggregates of non-polyglutamine sequences delivered to the nucleus are not toxic. Nuclear-localized aggregates of polyglutamine peptides are toxic regardless of peptide length. F-NLS-Q_{20} aggregates made *in vitro* and introduced into the nucleus of the cell are as toxic as Q_{42} aggregates. This supports the hypothesis that the repeat length dependence of disease risk in expanded CAG repeat diseases is related to the length dependence of aggregation efficiency. Once aggregates are formed, as governed by the biophysics of polyglutamine aggregation, and once these aggregates are delivered to the nucleus, any polyglutamine peptide aggregate is toxic. Monomeric polyglutamine sequences are toxic only to the

extent that they can aggregate during the course of the experiment and are targeted to the nucleus.

Cell death induced by the nuclear polyglutamine aggregates is a programmed cell death requiring caspase activation, which strongly suggested an apoptotic cell death pattern. The peptide, PGQ9($P^{2,3}$), which inhibits the recruitment of other polyglutamine sequences by polyglutamine aggregates, effectively reduces the cell death caused by nuclear aggregates. This further supports a recruitment mechanism for the cytotoxicity observed in our model system.

The new cell model system can be useful in testing new *in vitro* inhibitors at the cellular level during the drug discovery process. The ability to deliver such aggregates into different cell types should make possible a variety of studies on the biochemical details of cytotoxicity pathways.

Further studies to characterize the gene transcriptional profile changes in the system by DNA microarrays or differential RT-PCR are needed to confirm the proposed polyglutamine recruitment/apoptosis mechanism. The study described here was designed to evaluate the fundamental toxic properties of the polyglutamine sequence itself. The surrounding polypeptide sequences of actual disease-associated expanded polyglutamine proteins and their cellular expression profiles may modulate disease features through a number of mechanisms. It may be possible to conduct further experiments on aggregates of the disease proteins themselves to evaluate some of these effects. It will also be very valuable to conduct the same experiment in primary striatal neurons or cultured neuronal

tissues and analyze more subtle effects of the aggregates on neuronal function under non-toxic conditions.

CHAPTER 9

MATERIAL AND METHODS

Peptides and growing *in vitro* aggregates

Peptides

The peptides used in these experiments were prepared by solid-phase synthesis at the Keck Biotechnology Center of Yale University. The polyglutamine peptides contained flanking pairs of lysine residues to gain a net positive charge on the peptide at PH 7 to improve solubility and thus allow control over the aggregation reaction (Chen, Berthelier et al. 2001). A control peptide derived from the bacterial cold shock protein B (CspB-1) was also obtained (Gross, Wilkins et al. 1999). All peptides were synthesized with a fluorescein group on the N-terminus to facilitate microscopy and flow cytometry detection. Some peptides also included a nuclear localization signal peptide sequence (NLS) (Kalderon and Smith 1984) or nuclear export sequence (NES) (Mowen and David 2000). A pair of Gly residues was designed into the interface between the NLS (or NES) and the polyglutamine stretch to insure accessibility of the NLS (or NES) in the context of the aggregate. Peptides were used without purification, and HPLC analysis showed that the peptides contained some glutamine deletion impurities (Berthelier, Hamilton et al. 2001) as well as some peptides lacking the N-terminal fluorescein. These impurities do not appear to have influenced the results. In the cytotoxicity inhibitory study described

later, a polyglutamine peptide elongation inhibitor, discovered by mutational analysis in our laboratory, and named $PGQ_9(P^{2,3})$ (Thakur and Wetzel 2002), was used to test the effect of elongation inhibition on cytotoxicity. All the peptides used in the study are listed in Table 8.

Growing in vitro *aggregates*

Peptides were processed by first dissolving and rigorously disaggregating the lyophilized solid-phase synthesis product using an organic solvent treatment followed by ultracentrifugation of a pH 3 aqueous solution (Chen and Wetzel 2001). To grow aggregates, the pH 3 solution was neutralized to pH 7 using a buffered concentrated (10X PBS) and the peptide concentration was adjusted to 10 uM using 1X PBS as required. This solution was snap frozen in liquid N_2 and incubated for 48 hrs at –20 °C. This frozen reaction mixture was then thawed and centrifuged for 30 mins at 14,000 X g to collect the aggregates. HPLC analysis of the supernatant gives the amount of unaggregated material and allows calculation of an approximate value for the mass of aggregated peptide. It should be noted that the aggregation actually occurs when the frozen solution is stored at –20 °C, by the assistance of the process of freeze concentration (Chen, Berthelier et al. 2002). In the earlier experiments reported here, a 24 hrs preincubation of monomer was included before the snap-freezing step in the preparation of aggregates, but this was later eliminated without any effect on toxicity.

Sonication and filtration method to control the size of the aggregates

Aggregate size was modified by a two-step procedure involving sonication and membrane filtration. Aggregates were washed once with PBS using centrifugation at 14,000 X g to collect the aggregates. Aggregates were resuspended in PBS at a concentration of approximately 100 ug/ml. Sonication was accomplished with a probe sonicator at 0 °C in six pulses of 30 secs each. Sonicated aggregates were manually filtered through a 1.2 um Acrodisc syringe filter (Gelman Sciences). In some cases, the filtrate was then further filtered sequentially through a 0.8 um filter, a 0.45 um filter and a 0.22 um filter. Attempts to pass the aggregates through a 0.1 um filter failed due to high back pressure. The efficiency of filtration was determined by comparing the fluorescence signal of a suspension of filtered aggregates to that of non-filtered aggregates; triplicates of a 100 ul aliquot were diluted 4 times into wells of a microtiter plate and fluorescence monitored on the Wallac Victor 2 plate reader with excitation at 485 nm and emission at 535 nm. Determined by this method, about 50-80% of total sonicated aggregates were recovered from 1.2 um filter; 40-60% from 0.45 um filter; and 30-50% from 0.22 um filter. The aggregate suspensions were then adjusted to 10 uM concentration (monomer peptide equivalent) before applying them to cells. Aggregate preparation is summarized schematically in Figure 25.

Soluble peptide preparation

Monomers used in the studies were prepared by the standard method of processing the peptides as described above, without further growing the aggregates. After

ultracentrifugation, the pH 3 solution was neutralized to pH 7 using a buffer concentrate (10 X PBS) and immediately used to treat cells.

Liposome encapsulation

Liposome encapsulation of aggregates was accomplished using a commercially available pre-liposome formula (L-α-phosphatidylcholine and β-oleoyl-γ-palmitoyl cholesterol; Sigma L3906) according to the recommended procedure. Briefly, a 0.2 ml PBS suspension of aggregates was added to the dehydrated lipid film provided by the manufacturer, and the resulting suspension vortexed and then supplemented with 0.8 ml fresh basal medium.

Cell culture and treatment

Cell culture

Cos-7 cells were purchased from the ATCC (CRL-1651) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and antibiotics. PC-12 cells, kindly provided by Dr. Erik Schweitzer, were maintained in DMEM high glucose medium supplemented with 5% calf serum (Hyclone), 5% horse serum, and antibiotics. Medium was changed every other day; cells were plated at 100,000 / cm^2 density onto a 6-well plate the day before treatment with aggregates. For the LDH and MTS experiments, cells were plated in phenol red-free and serum-free medium for 24 hrs before the assay.

NGF differentiation of PC-12 cells

In some of the microscopy imaging and apoptosis studies, PC-12 cells were also differentiated into neuron-like cells by nerve growth factor (NGF) (Li, Cheng et al. 1999). In these studies, cells were plated at 50,000 / cm^2 density onto either 24-well plates or P-100 plates for 24 hours with medium containing 10% serum for proper growth and attachment. Then, the medium was replaced with basal medium without serum containing 50 ng/ml NGF (Sigma) for 24 – 48 hours to allow the PC-12 cells to properly differentiate into neuron-like cells before treatment with aggregates.

Treatment of the cells with aggregates or soluble peptides

For incorporation of unpackaged aggregates or monomers, cells were washed in 1X PBS and incubated with 10 uM aggregates or monomers for 24 hrs. For liposome packaged aggregates, washed cells were mixed with liposome-encapsulated aggregates (see above) for 4 hrs, after which the liposome mixture was removed from the plastic-attached cells by several washes with PBS. Fresh culture medium was then added, and the cells were incubated for another 20 hours.

Isolation of cell nuclei

Nuclei were isolated by a modification of the protocol of Hann *et al* (Hann, Dixit et al. 1994). Cells were lysed in NP-40 lysis buffer (0.6% NP-40, 0.1% BSA and 0.15 M NaCl) for 10 minutes on ice. Nuclei were collected by centrifugation at 500 X g for 5 minutes and washed twice, then re-suspended in PBS. Isolated nuclei were stained for

contrast with 50 ug/ml propidium iodide (PI) for 5 minutes before being examined by confocal microscopy.

Microscopy and flow cytometry analysis

Electron microscopy analysis

Aggregates were adsorbed onto carbon mica grids and negatively stained with 0.25% w/v potassium phosphotungstate solution. Images were collected on a Hitachi 500 electron microscope (Hitachi, Tokyo) at the microscopy center in Division of Biology, University of Tennessee, Knoxville.

Confocal microscopy

Cells were collected by first incubating with 1 ml per well of a solution of 0.5 mg/ml trypsin in a PBS-EDTA buffer (BioWhittaker, #17-161) until detachment was observed under the light microscope (about 1 min). At this point 2 ml per well of serum-containing culture medium was added and the suspension immediately transferred to a centrifuge tube. Cells were collected by centrifugation at 1,000 X g for 5 min, and then washed twice with PBS using the same centrifugation conditions. The washed cell pellet was resuspended in 1 ml PBS. The cell suspension was plated on a chamber slide (Nalgene Nunc International, Lab-Tek II Chamber slide system) and analyzed within 4-6 hours by confocal microscopy. Confocal microscopy was conducted using a Leica SP2 laser scanning confocal microscope.

Live-cell microscopic system

PC-12 cells were plated onto a special culture dish that can be heated evenly. In the culture medium, 50 ng/ml NGF was used to provide a better morphology of the cells; doubled concentration of antibiotics were used to limit the chance of contamination. The culture dish containing the cells was carefully transferred onto a special stage mounted onto the confocal microscope. The stage was connected to a temperature control system, which was set up to maintain 37 °C. The whole system, called Delta T Open Dish Live-microscopy System, was purchased from Bioptechs Inc. A special lens dipping into the culture dish was used to monitor the cells, and the images were collected automatically every 10-15 minutes by the computer. Once the focus was adjusted, the remaining area of the dish was covered with mineral oil to eliminate the evaporation of the culture medium and decrease the chance of contamination. A picture of the experimental setup is in Figure 40.

Flow cytometry analysis

Cells were injected into a FACScan (Becton-Dickinson, San Jose CA). Excitation at 488 nm allowed observation of both the fluorescein-tagged aggregates (FL-1, emission 530 nm) and the propidium iodide stained dead cells (FL-3, emission 650 nm). At least 10,000 events were collected for each sample and analyzed using Cell Quest software (Becton-Dickinson).

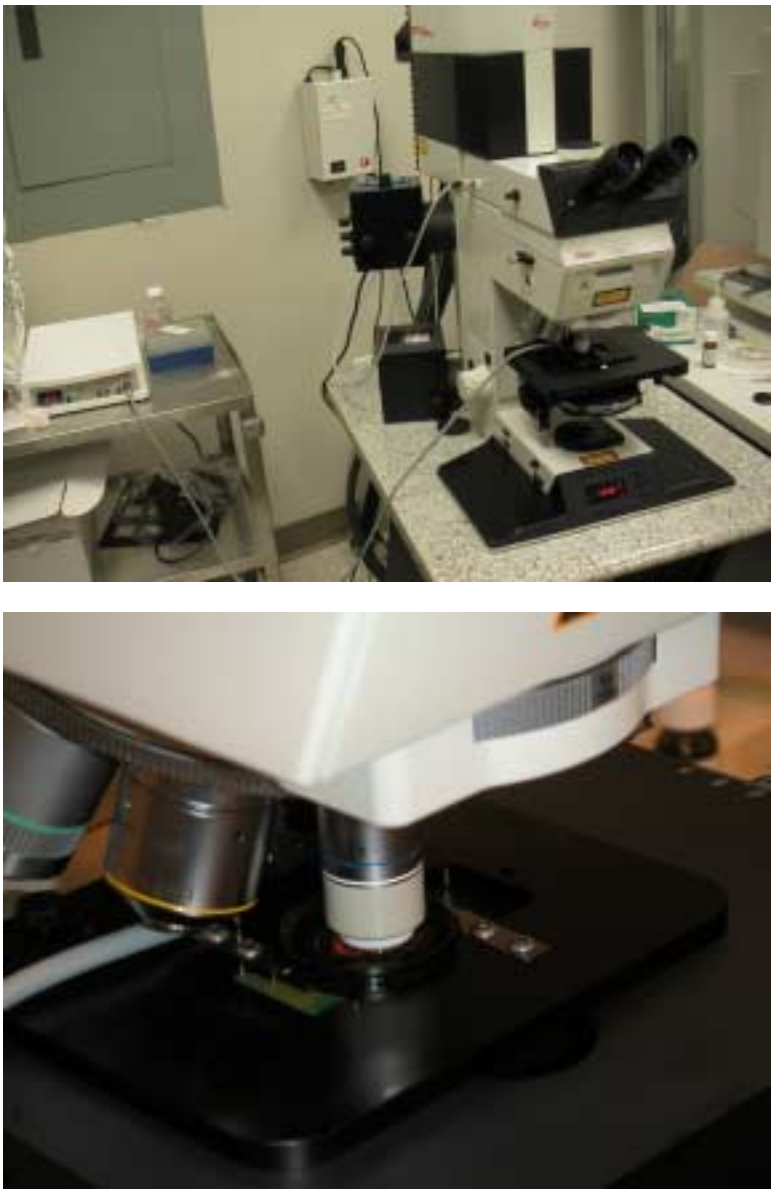


Figure 40. The configuration of the live cell microscopic system.

Cell Viability studies

Flow cytometry monitored PI incorporation

To assess cell viability, a suspension of washed cells after treatment with aggregates was stained with 50 ug/ml PI for 5-10 minutes and then injected into a FACScan as described above. Cell death was measured by the percentage of cells containing PI-stained nuclei, which indicates that cell membrane integrity was lost when the cells died.

LDH release assay

The lactate dehydrogenase (LDH) release assay (Decker and Lohmann-Matthes 1988) was used to assess cell death by measuring the activity of the cytosolic enzyme LDH released into the cell medium. LDH activity was quantified using a linked enzyme assay. In the oxidation of lactate to pyruvate by LDH, the NAD+ cofactor is reduced to NADH. NAD+ is regenerated from the NADH by the action of the enzyme diaphorase in the reduction of the INT tetrazolium ion to its corresponding formazan product. The red formazan product is then quantified in a spectrophotometer. The assay was conducted using the Promega CytoTox 96 assay kit (#G1780) with some modification of the manufacturer's protocol. After 24 hr incubation of the cells with polyglutamine aggregates, 200 ul of medium was removed and centrifuged for 5 min at 14,000 X g to remove aggregates and cell material. Three aliquots of 50 ul each of the supernatant were pipetted into microplate wells, followed by 50 ul per well of the diaphorase plus

substrates solution, and the plate incubated 30 mins in the dark at RT. The reaction was stopped by addition of 50 ul 1 M HOAc, and the absorbance read at 490 nm in a SpectraMax Plus UV-VIS microtiter plate reader (Molecular Devices, Inc.). The maximum LDH release of the cells was assessed by incubating a control plate of cells with 100 ul/ml of a 9% v/v Triton X-100 solution for 45 mins at 37 °C. A blank value was obtained by treating an aliquot of culture medium with the assay reagents; all LDH values were corrected by subtracting this blank value. Relative cytotoxicity was calculated by dividing LDH release for a particular experiment by the total LDH content of cells determined after Triton X-100 lysis. It should be mentioned that, in the LDH release assay, serum added to the culture medium contains a high LDH background, and this background varies with serum purchased at different times. Presumably, there is LDH in animal sera with the amount of LDH varying depending on the species and the health or treatment of the animal prior to the collecting serum. This high and variable background can be eliminated by growing cells in serum free media, which, however, introduces the disadvantage of greater cell death due to serum starvation.

MTS reduction assay

Cell death or dysfunction as indicated by diminished activity of mitochondrial dehydrogenases was assessed using the MTS assay, a modification of the standard MTT assay (Shearman 1999). Viable cells actively regenerating NADH and NADPH, were incubated with the MTS tetrazolium substrate (3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyl tetrazolium bromide) plus the electron coupling agent phenazine ethosulfate, carry out the

reduction of the tetrazolium to the corresponding formazan product, which can be quantified by its red color in a spectrophotometer. The procedure was carried out using the CellTiter 96 Aqueous One-Solution Cell Proliferation Assay kit (Promega #G3582) by a modification of the provided protocol. After 24 hr incubation of cells with aggregates, cells were incubated with 200 ul of assay solution to 1 ml of cell culture and the mixture incubated in a 24-well sterile microplate for 4 hr at 37 °C, 5% CO_2. The mixture was transferred to a microtube and centrifuged at 14,000 X g for 5 mins. The entire supernatant was then transferred to a 1 ml cuvet and the absorbance read in the cuvet port of the SpectraMax Plus UV-VIS microplate spectrophotometer (Molecular Devices). A maximal MTS reduction value was determined for control cells not otherwise treated, and a reagent blank was determined by treating culture medium alone with MTS reagents. Relative cell survival was determined by dividing the corrected A_{490} for a particular experiment by the corrected A_{490} for untreated cells.

Apoptosis studies

Western Blot for PARP cleavage detection

Poly (ADP-ribose) polymerase (PARP) is a 116 kD protein involved in normal DNA repair function (Smulson, Simbulan-Rosenthal et al. 2000). PARP is a substrate for the executive caspases (caspases 3, 7) during apoptosis (Lazebnik, Kaufmann et al. 1994). It is cleaved into an 85 kD carboxyl terminal fragment and a 25 kD amino terminal fragment by activated caspases (Lazebnik, Kaufmann et al. 1994). This is a widely used method to detect the activation of caspases and for studying apoptosis. Anti-PARP (Santa

Cruz Biotechnology, Inc) recognizing the C-terminus of PARP can detect both 116 kD and 85 kD fragments in a western blot. After 24-48 hours of treatment by aggregates, both floating and attached cells were collected and lysed in PARP lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors) by sonication for 30 s on ice (Xu, Shi et al. 2002). Total protein concentration in the cell lysate was determined by Micro BCA™ protein assay reagent kit (Pierce). An equal amount of total protein was subjected to electrophoresis on a 4-20% Tris/glycine SDS-PAGE gel and the gel electrotransferred to a polyvinylidene fluoride membrane (Invitrogen). The membranes were then probed with anti-PARP antibody, and visualized by ECL-Plus (Amersham Pharmacia Biotech).

Caspase inhibitor study

Z-VAD-FMK (Z-Val-Ala-Asp-CH2F) (Calbiochem) is a cell-permeable, general caspase inhibitor. It acts by binding to the active site of caspases and inhibiting its activity irreversibly, thereby also inhibiting apoptosis. PC-12 cells were plated in 24 well plates and treated with aggregates. Different amounts (0, 12.5 and 25 uM)) of Z-VAD-FMK was added to the cell medium and incubated for 24-48 hours. The LDH assay was used to study the difference in cell death between aggregate treated cells with or without added Z-VAD-FMK.

Protein synthesis inhibitors study

Actinomycin D (Act. D) is an anti-neoplastic antibiotic. It inhibits RNA polymerase activity by blocking its DNA binding site and therefore inhibits gene

transcription and stops new protein synthesis. Cycloheximide (CHX) is an antibiotic produced by *S. griseus*. Its main biological activity is inhibition of protein translation in eukaryotes resulting in inhibition of protein synthesis. It has been shown that both Act.D and CHX can act as inhibitors of programmed cell death, which requires new protein synthesis (Sperandio, de Belle et al. 2000). After 4 hours of treatment with aggregates (described as above), cells were treated with 1.0ug/ml Act.D or CHX by direct adding into the cell medium to see if they can inhibit the cell death observed in the LDH assay.

DNA fragmentation study

DNA laddering

After treated with aggregates, cells, both attached and floating, were collected and washed twice in PBS. The cells were lysed in a lysis buffer containing 10nM EDTA, 50 mM Tris PH 8, 0.5% sodium lauryl sarcosine, 0.5mg/ml proteinase K and the total cell DNA was extracted by phenol-chloroform extraction, precipitated by addition of sodium acetate and centrifuged at 14,000rpm for 10 min at 4 °C. The DNA pellet was then washed with ethanol and resuspended in TE buffer (10mM Tris PH 7.4 and 1mM EDTA). 15 ug of DNA was loaded on a 1% agarose gel. The gel was stained with ethidium bromide and visualized with UV.

DNA fragmentation ELISA assay

The ELISA assay was purchased from Roche Applied Science as a kit. The procedure is as suggested by the manufacture. Cells were plated in a 24 well plate at 5 X

10^4/ well and treated with different aggregates for 24 hours. Then cells were collected and washed in PBS, then lysed in the provided lysis buffer for 30 min at room temperature. The cell lysate was centrifuged at 200 g for 10min to collect the supernate containing the fragmented DNA. 20ul of the supernate was transferred to a streptavidin-coated plate. 80ul of the mixture containing the anti-histone-biotin and anti-DNA-POD (peroxidase conjugated) were added to the sample in the microtiter plate. The plate was covered and incubated on a shaker for 2 hours at room temperature. After incubation, the solution was removed and the plate was washed thoroughly with the incubation buffer. Then ABTS (2, 2'- Azinobis[3-ethylbenothiazoline-6-sulfonic acid]-diammonium salt), which is a substrate for peroxides, was added, which yields a green end product measuarable at 405 nm in a SpectraMax Plus UV-VIS microtiter plate reader (Molecular Devices, Inc.)

Annexin V binding study

Cells were treated with aggregates for 16 hours and then collected and washed twice in PBS. Then the cells was resuspended in Annexin-Binding buffer (10mM HEPES, 140 mM NaCl and 2.5mM $CaCl_2$, PH 7.4). 10 ul Annexin V –Alexa Fluor 647 conjugate (Molecular Probes) was then added to the 100 ul of cell suspension and incubated for 15 minutes at room temperature. Cells were then washed with Annexin-Binding buffer twice and dipped onto a slide to be observed under confocal microscope.

Inhibitor studies

The polyglutamine aggregation elongation inhibitor $PGQ_9(P^{2,3})$ (listed in Table 3) was pre-incubated with cells at the concentration range from 0 to 100 uM for 24 hours before treatment with aggregates or monomers. Cell death was evaluated by LDH assay after 24-48 hours incubation with aggregates.

Statistics

Three to eight independent studies (depending on the aggregate tested), including aggregate preparation and cell treatment, were conducted for flow cytometric analysis of cell viability. Two to four independent experiments were conducted for LDH and MTS assays. Bar graph data are expressed as the mean +/- the standard error. Statistical significance was analyzed by one-way ANOVA using the software package SPSS 10.0 (SPSS, Chicago IL). A p value ≤ 0.001 is considered significant.

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VITA

Wen Yang was born in Liaoning, China in 1974. In July 1998, she graduated from China Medical University, China with a Medical degree in Clinical Medicine. Then she started her residency in the Department of Medicine in the affiliated hospital of Dalian Medical University. She married in December 1998. In January 1999, she came to USA to pursue her Ph.D. degree in the Department of Comparative and Experimental Medicine, Graduate School of Medicine, the University of Tennessee at Knoxville. After the acceptance of this dissertation, she will be leaving UT with her Ph.D. degree.